CONSTRICTOR PROSTANOID-POTENTIATED VASCULAR CONTRACTION: REGULATION OF ENDOTHELIAL AND VASCULAR SMOOTH MUSCLE MECHANISMS BY ESTROGEN

A Dissertation

by

MIN LI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Veterinary Physiology

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Approved as to style and content by:

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ABSTRACT

Constrictor Prostanoid-Potentiated Vascular Contraction: Regulation of Endothelial and Vascular Smooth Muscle Mechanisms by Estrogen. (May 2004)

Min Li, B.M.S., Beijing Medical University

Chair of Advisory Committee: Dr. John N. Stallone

The objectives of this research were to elucidate the involvement of constrictor prostanoids in the vascular reactivity to vasopressin (VP) and the role of estrogen in the regulation of the constrictor prostanoid mechanism in the female rat. Aortas obtained from male, intact (InT)-, ovariectomized (OvX)- and OvX + estrogen-replaced (OvX+Est)-female rats were studied. Contractile responses to VP were examined in the presence of nonselective and selective cyclooxygenase (COX) inhibitors. Basal and VP-stimulated release of thromboxane A_2 (TxA_2) and prostacyclin (PGI_2) from the aortic wall were measured. Concentration-response curves to exogenous TxA_2 were also obtained. To elucidate the regulatory effects of estrogen on the constrictor prostanoid pathway, the expression of COX-1, COX-2, thromboxane synthase (TxS) and thromboxane receptor (TP) mRNA were detected by reverse transcription-polymerase chain reaction (RT-PCR). Further, immunohistochemistry was employed to determine COX-1, COX-2 and TxS protein expression in aortic endothelium and vascular smooth muscle.

The major findings of this research are that: 1) The contractile responses of the female rat aorta to VP were enhanced by COX-2-mediated production of constrictor

prostanoids (PGH_2/TxA_2), and this mechanism is potentiated by estrogen; 2) Vascular reactivity to exogenous TxA_2 was higher in the female than in the male rat aorta, and OvX attenuated and estrogen replacement therapy restored vascular reactivity to TxA_2 in the female aorta; 3) VP-stimulated release of endogenous TxA_2 and PGI_2 were higher in the female than in the male rat aorta, and OvX attenuated and estrogen replacement therapy restored VP-stimulated release of these endogenous prostanoids by the female aorta; and 4) The expression of COX-2 and TxS mRNA and protein, and the expression of TP mRNA were higher in InT-female than in male, and were reduced by OvX and restored by estrogen replacement therapy.

In conclusion, estrogen potentiated contractile responses of the female rat aorta to VP by upregulating the expression of COX-2, TxS and TP; thereby enhancing VP-induced release of TxA_2, as well as the vascular reactivity to endogenous TxA_2.

DEDICATION

This work is dedicated to my daughter, Sophia Chen. She was born in College Station while I was still doing my research. Her birth brought whole new meaning to my life. I hope she will be healthy and happy when she grows up. Hopefully, knowledge gained from this work can be useful for all the women in the world.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. John N. Stallone for his guidance and support, and to my committee members, Dr. Timothy A. Cudd, Dr. Jeremy S. Wasser, Dr. Janet L. Parker, for their patient assistance and service on my advisory commitee.

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CHAPTER I

INTRODUCTION

Epidemiological studies have revealed that the incidences of cardiovascular diseases, principally coronary artery disease and hypertension, are lower in premenopausal women than in men, but that after menopause, these diseases increase in women to levels similar to those of men (6, 12, 85, 223). These observations suggest that female gonadal steroid hormones may exert protective effects on the cardiovascular system. Human clinical and animal experimental studies have demonstrated that estrogen decreases serum cholesterol levels (260), stimulates growth of vascular endothelium (165), inhibits growth of vascular smooth muscle (VSM) cells (21, 130), and induces release of the local vasodilators such as nitric oxide (NO) (98, 259) and prostacyclin (PGI_2) (171). However, large-scale hormone replacement therapy (HRT) studies do not show an overall beneficial effect of estrogen in postmenopausal women (94, 95, 105, 113, 215). In fact, in the first year of HRT (combination estrogen and progesterone), the incidence of deleterious cardiovascular events increased. Further, premenopausal women are more likely to develop diseases that are primarily vascular in origin, such as primary pulmonary hypertension, Raynaud's disease and some forms of migraine headache. These data suggest that estrogen also may exert deleterious effects on the cardiovascular system. Animal studies suggest that estrogen enhances vascular

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reactivity to vasopressin (VP), phenylephrine (PE), noradrenaline (70, 168), and that cyclooxygenase (COX) metabolites contribute to the estrogen-mediated increases in vasoconstriction (70, 159, 225). Epidemiological and clinical data suggest that vascular thromboxane A_2 (TxA_2) may be the common mechanism underlying the vascular diseases more prevalent in women (13, 37, 208). Therefore, in the present studies, the role of estrogen in the regulation of arachidonic acid metabolism and vasoconstrictor mechanisms will be examined. These studies will focus on the effects of estrogen on constrictor prostanoid-potentiated VSM reactivity to VP and interactions between VSM and the vascular endothelium.

These doctoral studies will further clarify male-female differences in vascular function and elucidate the underlying mechanisms as well as the controversial effects of estrogen on vascular function. These studies also may lead to more effective treatments for hormone-dependent cardiovascular diseases in women.

Table 1 summarizes the major abbreviations used in this dissertation.

Table 1. Major abbreviations used in dissertation.

Abbreviation	Explanation	Abbreviation	Explanation
AA	Arachidonic acid	AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme	Ach	Acetylcholine
AF	Activation function	AT	Angiotensin receptor
ANG	Angiotensin	BK	bradykinin
COX	Cyclooxygenase	DAG	diacylglycerol
E	Epinephrine	EDHF	Endothelium-derived hyperpolarizing factor
EP	Prostaglandin E_2 receptor	ER	Estrogen receptor
ERE	Estrogen response element	ET	Endothelin receptor
F	Female	GC	Guanylyl cyclase
HDL	High-density lipoprotein	HERS	Heart and Estrogen/ progestin Replacement Study
HRT	Hormone replacement therapy	Indo	Indomethacin
InT	Intact	IP	Prostacyclin receptor
IP_3	Inositol 1,4,5-triphosphate	KHB	Krebs-Henseleit-bicarbonate solution
M	Male	MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction	NE	Noreprinephrine
NO	Nitric oxide	NOS	Nitric oxide synthase
NS	NS-398	OvX	Ovariectomy
OvX+Est	Ovariectomy plus estrogen replacement	PES	Prostaglandin endoperoxide synthase
PG	Prostaglandin	PGH_2	Prostaglandin endoperoxide
PGI_2	Prostacyclin	PIP_2	Phophatidylinositol (4,5)-bisphosphate
PKA	Protein kinase A	PLC	Phospholipase C
RAS	Renin-angiotensin system	RIA	Radioimmunoassay
RT-PCT	Reverse transcription-polymerase chain reaction	SERM	Selective estrogen receptor modulator
SHR	Spontaneous Hypertensive Rats	TP	Thromboxane receptor
TxA_2	Thromboxane A_2	TxB_2	Thromboxane B_2
TxS	Thromboxane synthase	VP	Vasopressin

CHAPTER II

LITERATURE REVIEW

2.1. Sexual dimorphism in vascular diseases

Epidemiological studies have established that prominent sexual dimorphism exists in a variety of human vascular diseases. Pulmonary hypertension, migraine headache, and Raynaud's Disease all occur more frequently in premenopausal women than in men. These findings suggest that female gonadal steroid hormones may exacerbate certain vascular diseases.

Pulmonary hypertension is diagnosed as a variety of conditions that lead to the elevation of pulmonary arterial pressure (252). The cause of the disease is unknown. In young and middle-aged women, the incidence of primary pulmonary hypertension is 1.7-3.5 times that in men (73). These observations suggest that female gonadal steroid hormones may contribute to the pathogenesis of this disease. This hypothesis is supported by reports that women taking oral contraceptives have a higher risk of developing pulmonary hypertension (107, 128).

Migraine headache is characterized by repeated or recurrent headache, associated with temporary increases in the tone of the scalp and neck arteries that supply blood and oxygen to the brain (207). Migraine headaches affect about 6 out of every 100 people, and are a common type of chronic headache. The ratio of women to men who develop migraine headache is 3:1 (194), again implicating female gonadal steroid hormones in this disease.

Raynaud's disease is a disorder that affects blood vessels in the fingers, toes, ears, and nose. This disorder is characterized by episodic attacks, called vasospastic attacks, which cause the blood vessels in these regions to constrict abnormally. Although estimates vary, recent surveys show that Raynaud's phenomenon may affect 5 to 10 percent of the general population in the United States. Women are more likely than men to develop the disorder and the incidence in female varies from 4 to 7.1 times that in male (14, 25, 87, 251).

Sex differences observed in these vascular diseases suggest that female gonadal steroid hormones may exert deleterious effects on the vasculature that contribute to the development of these disorders; however, other observations suggest that female gonadal steroid hormones also exert protective effects on the cardiovascular system. For example, premenopausal women have a low incidence of coronary heart disease, and after menopause, the incidence increases to levels observed in men (6, 12, 85, 223). Interestingly, the Heart and Estrogen-progestin Replacement Study (HERS) showed no overall benefit of estrogen replacement therapy. In fact, untoward cardiovascular events increased significantly in the first year of the study (94, 95, 105). Another study that compared 83 women with acute myocardial infarction (MI) who were younger than 46 years old and 154 controls showed a strong positive association between MI and oral contraceptive or other estrogen use (111). The use of oral contraceptives also increases the risk of thrombosis (17, 52). The controversy surrounding observations of sexual dimorphism in cardiovascular diseases has stimulated investigators to study sexual dimorphism in vascular function, the effects of female gonadal steroid hormones on the

cardiovascular system, and the underlying mechanisms of ovarian steroid hormone actions on the cardiovascular system.

2.2. Mechanisms of vascular reactivity

The overall mechanisms of VSM contraction will be reviewed first, followed by an overview of the mechanisms that regulate vascular tone. This information will form the basis for a discussion of the current understanding of male-female differences in vascular reactivity.

2.2.1. Mechanism of vasoconstriction

Contraction of VSM ultimately involves an increase in the intracellular cytosolic free Ca^{2+} concentration (112). Neural, humoral or other stimuli can cause Ca^{2+} influx from the extracellular fluid via sarcolemmal membrane Ca^{2+} channels and/or cause internal Ca^{2+} release from the sarcoplasmic reticulum. In turn, the increased cytosolic Ca^{2+} binds with the regulatory protein calmodulin, and the calcium-calmodulin complex can then activate the enzyme myosin light-chain kinase, which phosphorylates myosin. Phosphorylated myosin then interacts with actin to form crossbridges and cause VSM shortening (contraction). Relaxation is accomplished by active removal of Ca^{2+} from the cytosol by extrusion across the plasma membrane and by sequestration back into the sarcoplasmic reticulum.

2.2.2. Regulation of vascular tone

Blood vessel diameter is regulated by numerous mechanisms and mediators, including neural, circulating humoral substances, VSM autoregulation, local metabolites, and local humoral substances produced by the vascular endothelium and/or VSM.

2.2.2.1. The role of vascular endothelium in regulating vascular tone

Vascular endothelial cells produce many vasoactive substances that cause vasoconstriction or vasodilation of the underlying smooth muscle. The most important endothelium-derived vasodilator is nitric oxide (NO). Other important vasodilators include prostacyclin, bradykinin (216), and endothelium-derived hyperpolarizing factor (EDHF) (1, 15) (Fig. 1).

NO is formed from the guanidine nitrogen of L-arginine by the enzyme NO synthase (NOS). There are three isoforms of NOS: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). eNOS is Ca^{2+}-calmodulin dependent and constitutively expressed in vascular endothelium, cardiac myocytes, blood platelets and other tissues. NO activates guanylyl cyclase (GC) in VSM cells, leading to an increase in intracellular cyclic GMP (cGMP) level, which causes vasodilation (210). The synthesis and release of NO by eNOS can be stimulated by sheer stress on the vascular endothelium, increased intracellular Ca^{2+} in endothelium, and vasoconstrictor or vasodilator agonists that interact with the endothelium. Substances such as VP, acetylcholine (ACh), catecholamines and substance P all interact with their respective endothelial receptors to

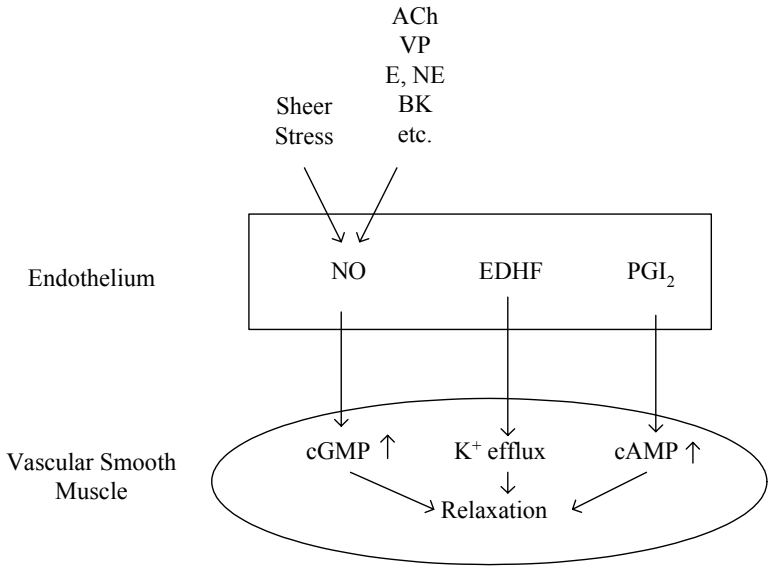

Fig. 1. Endothelium-derived vasodilator factors. Sheer stress and various chemical and humoral substances evoke the release of nitric oxide from endothelium. NO then increases cGMP level in the vascular smooth muscle (VSM) cell, which causes vasodilation. The endothelium also produces prostacyclin, which increases cAMP level in the VSM cell, which causes vasodilation. Endothelium-derived hyperpolarization factor (EDHF) is another vasodilator, which acts by opening potassium channels. NO, nitric oxide; ACh, acetylcholine; VP, vasopressin; E, epinephrine; NE, norepinephrine; PGI_2, prostacyclin; EDHF, endothelium-derived hyperpolarization factor; BK, bradykinin.

activate eNOS and mediate agonist-induced release of NO (210, 216). Under normal conditions, NO is a major determinant of local vascular tone (163).

Endothelium-derived vasoconstrictors include endothelin-1, prostaglandin endoperoxide (PGH_2), TxA_2, angiotensin II (ANG II), and superoxide anions (120, 244) (Fig. 2).

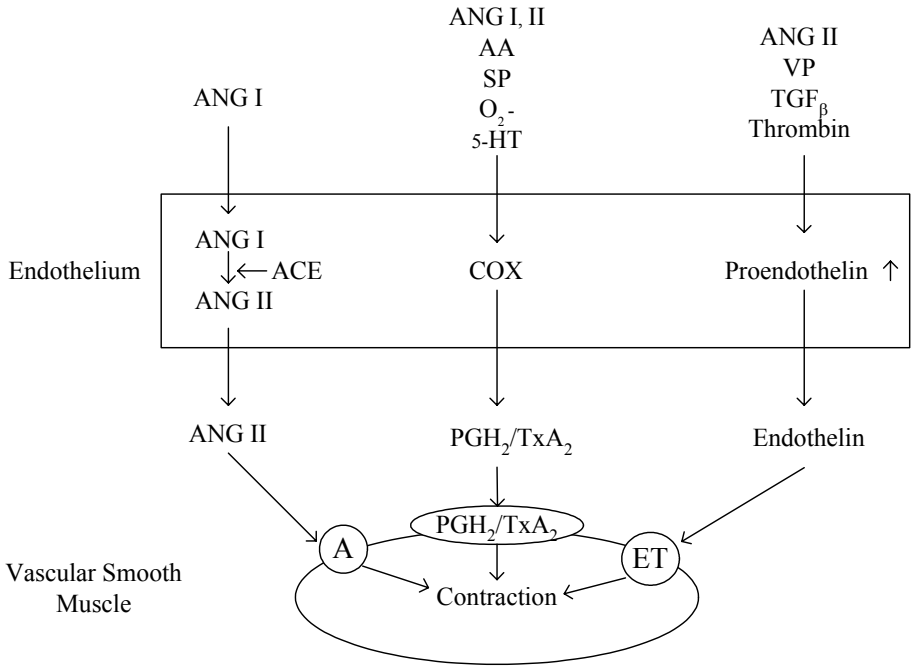

Fig. 2. Endothelium-derived vasoconstrictor factors. Angiotensin converting enzyme (ACE) converts angiotensin I (ANG I) to angiotensin II (ANG II), which acts on angiotensin receptor (A) and cause vasoconstriction. ANG I, ANG II, arachidonic acid (AA), superoxide anion ($O_2^{\cdot -}$), 5-hydroxytryptamine (5-HT) and substance P (SP) can activate the cyclooxygenase (COX) pathway to produce prostaglandin H_2 (PGH_2) and thromboxane A_2 (TxA_2), which act on their receptor (PGH_2/TXA_2 receptor) and cause vasoconstriction. Production and secretion of endothelin can be activated by ANG II, vasopressin (VP), transforming growth factor beta (TGF_β) and thrombin. Endothelin then interacts with its VSM receptor (ET) to cause vasoconstriction.

Endothelial dysfunction appears to be involved in the pathogenesis of a number of cardiovascular diseases. It was reported that during the development of cardiovascular diseases the synthesis and release of NO is blunted (31, 139, 238), while the production of other relaxing and contracting factors also may become imbalanced (156, 205). For example, the vasodilatory response of the brachial artery to ACh is reduced in patients with essential hypertension compared to normotensive controls, and infusion of the NOS inhibitor L-NMMA into the brachial artery of hypertensive subjects produces a

significantly smaller decrease in basal blood flow compared to normotensive controls, suggesting that basal release of NO is deficient in hypertensive patients (185). Similarly, in patients with pulmonary hypertension, the ratio of TxB_2 to 6-keto-$PGF_{1\alpha}$ in plasma is increased (13). Further, radioimmunoassay studies showed that 24-hour excretion of 11-dehydro-thromboxane B_2 (a stable metabolite of TxA_2) is increased in patients with primary pulmonary hypertension and secondary pulmonary hypertension, as compared with normal controls, whereas the 24-hour excretion of 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ (a stable metabolite of prostacyclin) is decreased in hypertensive patients (37).

2.2.2.2. Circulating vasoactive substances

Vasopressin (VP) is synthesized in the magnocellular neurosecretory cells in the paraventricular and supraoptic nuclei of the anterior hypothalamus (74, 199). It is stored in the posterior pituitary until the osmoreceptors in the anteroventral portion of the third ventricle sense an increase in plasma osmolality and activate the release of this hormone (24). Reductions in arterial blood pressure and/or blood volume are also potent stimuli for the release of VP. There are two major groups of receptors that can effect changes in VP release in response to changes in blood volume and arterial pressure, i.e., stretch receptor in the atria of the heart, which respond to changes in atrial volume; and the arterial baroreceptors, which respond the sufficient changes of blood volume (78). VP is also known as antidiuretic hormone. There are three subtypes of VP receptors: V_{1a}, V_{1b}, and V_2 (23). The V_2 VP receptor, found principally in the kidney, activates adenylyl cyclase (AC) via stimulatory G-protein (G_s) and promotes the insertion of aquaporin-2

water channels into the luminal surface of the renal collecting tubules, thereby increasing water permeability and reabsorption of water from the urine (53, 57). The V_{1b} receptor, located on corticotroph cells of pituitary, activates phospholipase C (PLC) and stimulates the release of adrenocorticotropin (ACTH) from corticotroph cells of the anterior pituitary (3). The V_{1a} VP receptor, located principally on VSM cells, activates phospholipase C by interaction with a membrane-associated G protein ($G_{q/11}$) and thereby causes vasoconstriction (Fig. 3).

Under normal conditions, the vasoconstrictor effect of VP does not participate in the daily regulation of vascular tone; rather, it appears to function mainly in short-term (acute) and long-term (chronic) control of blood pressure in hypovolemic/hypotensive states such as hemorrhage or dehydration (145).

Angiotensin II (ANG II) is produced both systemically and locally within the vascular wall. Systemically, angiotensinogen is synthesized in liver, released into circulation, and interact with renin that is released from the kidney into the circulation. Renin catalyzes the conversion of angiotensinogen into angiotensin I (ANG I). Angiotensin converting enzyme (ACE), present in the lung and other vascular tissue, in turn then converts circulating ANG I into ANG II. Several studies have confirmed that renin, angiotensinogen, ACE and ANG II are present in blood vessel walls (60, 61). In the human heart, ANG II is also formed by Chymase, an ACE-independent pathway (243). Systemic ANG II plays roles in both short-term (acute) and long-term (chronic) regulation of arterial blood pressure. Its production is increased in response to

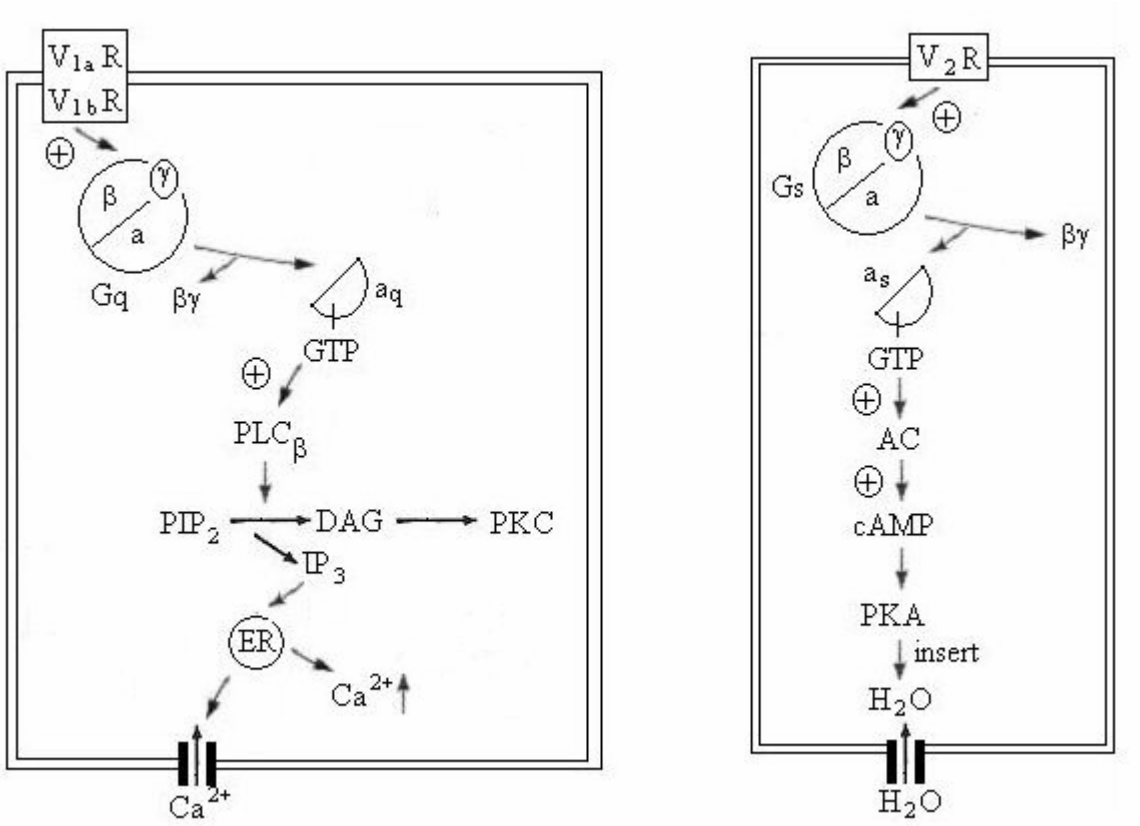

Fig. 3. Coupling mechanisms of the V_1 and V_2 vasopressin receptors (V_1R and V_2R, respectively). The left-hand figure represents a hypothetical cell containing V_1R (vascular smooth muscle cell) and the right-hand figure represents a hypothetical cell containing V_2R (renal collecting tubule cell). Binding of V_1R by VP promotes the dissociation of Gq-GTP. The α_q-subunit stimulates PLC_β activity and then promotes hydrolysis of PIP_2, which increases the intracellular levels of DAG and IP_3; IP_3 in turn stimulates the IP_3R that resides in the membrane of the ER and promotes Ca^{2+} release from intracellular stores. Gq also activates receptor-opened calcium channel (ROC). The right-hand figure summarizes the V_2R associated signaling in the principal cells of the renal collecting duct; the occupied receptor promotes dissociation of Gs-GTP into it's $\beta\gamma$ and α subunits; the α subunit stimulates AC activity which subsequently increases cAMP level. cAMP then activates PKA and the insertion of the preformed water channel into the luminal surface of the cell. Abbreviations: AC, adenylyl cyclase; DAG, diacylglycerol; ER, endoplasmic reticulum; PIP_2, phosphatidylinositol (4,5)-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC_β, phospholipase C_β.

hypotension, hypovolemia, hemorrhage, and severe heart failure. In addition to the circulating renin-angiotensin system (RAS), local, tissue-generated angiotensin is also reported to exist, and may contribute to long-term cardiovascular homeostasis (143, 230).

Angiotensin not only vasoconstricts blood vessels acutely, but also stimulates VSM cell growth, and appears to participate in the pathogenesis of vascular diseases such as hypertension, atherosclerosis and restenosis (62). There are two subtypes of angiotensin receptors (AT): AT_1 and AT_2. The AT_1 receptor activates PLC and produces inositol 1,4,5-triphosphate (IP_3) and DAG. IP_3 increases cytosolic Ca^{2+} concentration by stimulating the release of Ca^{2+} from the endoplasmic reticulum. The AT_1 receptor is responsible for vasoconstriction, aldosterone and VP release, sodium and water retention and sympathetic facilitation (51, 118). The AT_2 receptor is expressed at very high levels in the developing fetus, whereas its expression is very low in the cardiovascular system of the adult. The expression of the AT_2 receptor can be modulated by pathological states associated with tissue remodeling or inflammation. AT_2 receptor stimulation inhibits cardiac fibroblast growth and extracellular matrix formation and exerts a negative chronotropic effect (102). The signaling mechanism of the AT_2 receptor has not been well defined, but at least involves the activation of protein tyrosine phosphatase (102). Angiotensin converting enzyme inhibitor (ACEI) and angiotensin receptor antagonists are widely used to treat cardiovascular diseases (56, 72).

2.2.3. Synthesis, metabolism and function of prostaglandins

2.2.3.1.Arachidonic acid metabolism

Arachidonic acid (AA) is the most common fatty acid present in the phospholipids of the mammalian cell membrane. It is released from cell membrane phospholipids by the enzyme phospholipase A_2 and diacylglycerol (DAG) lipase (150). The main routes of metabolism of AA are (Fig. 4): (1) the cyclooxygenase (COX) pathway, which produces the prostaglandins (PGs); (2) the lipoxygenase pathway, which gives rise to the leukotrienes and several hydroperoxides (HPETEs); and (3) the cytochrome P-450 pathway, which forms the P450-eicosanoids (33, 162, 176).

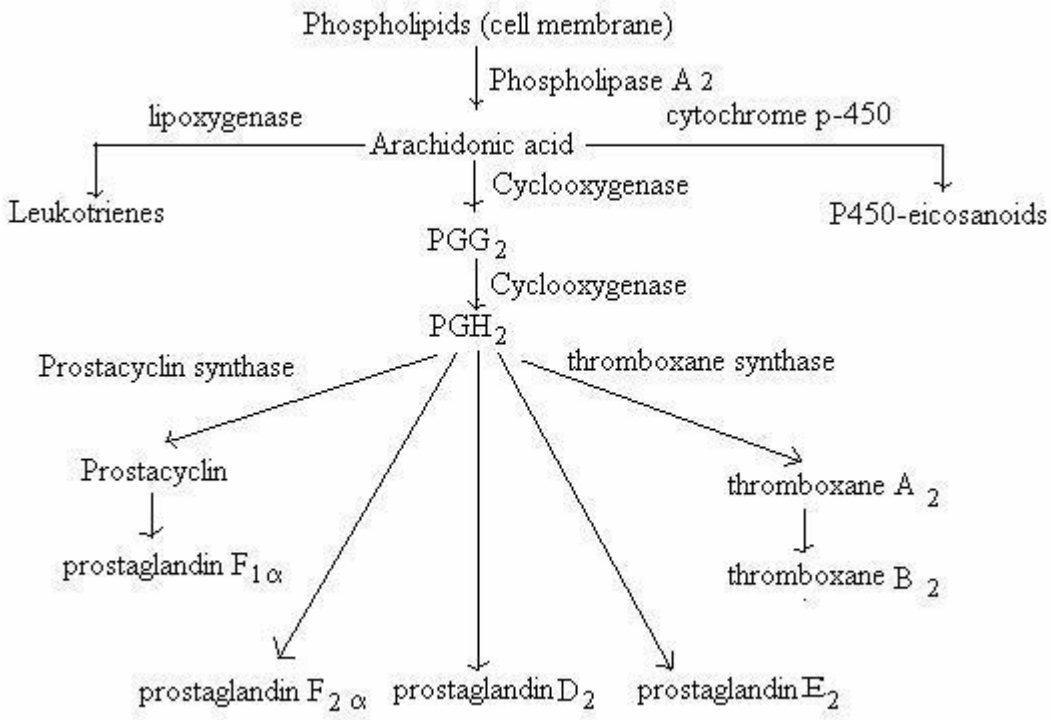

Fig. 4. The metabolic pathway of arachidonic acid.

The products of AA metabolism, especially via the COX and cytochrome P-450 pathways, play important roles in regulating the cardiovascular system. For example, various PGs function either as vasodilators or vasoconstrictors, and either as pro-coagulant or anti-coagulant substances, to regulate vascular tone and the maintenance of homeostasis (162, 176). Further, cytochrome P-450 mediates the release of EDHF, which cause vasodilation and therefore regulates vascular tone, especially in the coronary and renal arteries. In contrast, 20-hydroxyeicosatetraenoic acid (20-HETE), another cytochrome P-450 product, is a vasoconstrictor, particularly in the kidney (69, 201, 269).

2.2.3.2. Cyclooxygenase

In 1991, it was confirmed that there are two isoforms of COX: COX-1, and COX-2 (137, 264). They are encoded by different genes (131, 132). COX-1 is the constitutive form of the enzyme, and is present in many cells. In platelets, the only detected isoform is COX-1 (245). The analysis of the promoter of the human COX-1 gene suggests that COX-1 is a housekeeping gene (255). PGs produced by COX-1 regulate basal cell activities, such as platelet-dependent homeostasis, gastric mucosal integrity, and regulation of renal blood flow (219). In contrast, COX-2 was previously considered to be an inducible form, present at very low or undetectable levels in most quiescent cells, and strongly induced mainly by cytokines, growth factors, and tumor promoters (46, 154, 214). Recent studies, however, reveal that COX-2 is also constitutively expressed in some tissues such as kidney, brain, spinal cord, lung and

heart, and is responsible for regulating tissue functions under various physiological states (180, 186, 254).

Some studies suggest that COX-2 may not just augment the biosynthesis mediated by COX-1; rather it may have different functions independent of COX-1. Murakami et al. showed that mouse bone marrow-derived mast cells expressed both COX-1 and COX-2 at the same time, but IgE-stimulated release of PGD_2 was coupled to COX-1 activity, while cytokine-induced PGD_2 was coupled to COX-2 activity (169). In another study, Reddy et al. showed that in murine fibroblasts and macrophages, endotoxin stimulated the release of AA, but constitutive COX-1 could not convert this released AA into PGs; rather, the conversion required the expression of COX-2 (195). These studies suggest different functions of COX-1 and COX-2. This hypothesis is supported by evidence that the enzyme concentration and distribution differ. Although COX-1 and COX-2 are both present in endoplasmic reticulum and nuclear envelope, COX-2 staining was twice as concentrated in the nuclear envelope as in the endoplasmic reticulum (166). Recently, it was reported that COX-2-derived PGs act in the central nervous system to produce hyperalgesia, potentiate renin secretion in kidney, and modulate vascular tone and thrombosis in the cardiovascular system. (96, 186, 254, 266).

The catalytic capacities of both COX-1 and COX-2 are under physiological control. AA is transformed into the prostaglandin endoperoxide PGG_2 by the enzyme prostaglandin endoperoxide synthase (PES), the alternate chemical name for the COX enzyme. PES has both COX and peroxidase activities. The COX activity in both COX-1 and COX-2 is inherently latent and its activation requires interaction with the

hydroperoxides. The requirement for hydroperoxide-induced activation differs between COX-1 and COX-2. COX-2 requires much lower levels of hydroperoxide than does COX-1 (138). For example, Capdevila et al. demonstrated that only COX-1 activity was inhibited by reduced glutathione (GSH), which decreases the availability of the hydroperoxide activator (32).

The differences in the expression, intracellular localization, activation and function between COX-1 and COX-2 provide some possible explanations of the effects of estrogen on the expression of these enzymes (see below).

2.2.3.3. Prostaglandin metabolism

The COX activity of PES transforms AA into prostaglandin endoperoxide PGG_2, and the peroxidase activity further transforms PGG_2 into endoperoxide PGH_2. In cultured fibroblasts, immunochemical studies show that PES is present in the endoplasmic reticulum and the nuclear membrane, but not in the plasma membrane or the mitochondrial membrane (200).

Several enzymes then further convert PGH_2 into different prostaglandin metabolites. Thromboxane synthase (TxS) converts PGH_2 into TxA_2; prostacyclin synthase converts PGH_2 into prostacyclin (PGI_2); and other isomerases convert PGH_2 into the various PGs (PGD_2, PGE_2, $PGF_{2\alpha}$) (162, Fig. 4). PGG_2, PGH_2, TxA_2 and PGI_2 are chemically unstable ($t_{1/2}$ = 30 sec), and under physiological conditions, they are rapidly degraded into inactive metabolites. Other PGs (PGD_2, PGE_2, $PGF_{2\alpha}$), although more stable, are still metabolized rather quickly ($t_{1/2} \leq 60$ sec) (84, 90, 175, 209, 234).

Thus, prostanoids (PGs and thromboxane) are likely to be active only at the sites of their production, or in the immediate vicinity.

2.2.3.4. Functions of prostaglandins

2.2.3.4.1. Thromboxane

TxA_2 is the metabolite of PGH_2 mainly produced from blood platelets (141). TxA_2 spontaneously hydrolyzes to its inactive product thromboxane B_2 (TxB_2). TxA_2 acts on the PGH_2/TxA_2 receptor (TP) to induce vasoconstriction and platelet aggregation.

TP is a G protein-coupled rhodopsin-type receptor with 7 transmembrane domains (97). TxA_2 acts on its receptor in VSM to activate G_q-protein, which activates phospholipase C, leading to inositol 1,4,5-triphosphate (IP_3) production, and subsequent mobilization of intracellular Ca^{2+}, causing vasoconstriction. TxA_2 receptor-mediated contraction of VSM also involves influx of extracellular Ca^{2+} via L-type and non-L-type calcium channels (108, 197). Since TxA_2 and PGH_2 share the same receptor (157, 248), both PGH_2 and TxA_2 exert similar effects via the same receptor. Even in the absent of TxA_2, for example in the presence of TxS inhibitor, PGH_2 will still activate TP and modulate cardiovascular function such as platelet aggregation or vasoconstriction.

TxA_2 may contribute to the pathogenesis of some cardiovascular diseases. Walinsky et al. showed that during the early stages of acute myocardial infarction (AMI), TxA_2 production increased in AMI patients and in left anterior descending artery-occluded dogs (253). In newborn pigs exposed to chronic hypoxia for 3 days, the

constrictor response of pulmonary arteries to ACh was abolished by the TxS inhibitors dazoxiben or feregrelate, or the TP antagonist SQ-29,548, which suggests the involvement of TxA_2 and/or PGH_2 in the development of this model of pulmonary hypertension (69). Monocrotaline pyrrole (MCTP) causes pulmonary endothelial cell injury and pulmonary hypertension in rats. It was reported that MCTP treatment increased the release of TxB_2 from isolated rat lungs, thereby suggesting the involvement of TxA_2 in the development of pulmonary hypertension (76). Similarly, in the rabbit pulmonary artery, AA and methacholine both caused the contraction of the artery as well as the release of TxA_2 and PGI_2. Indomethacin (COX inhibitor), dazoxiben (TxS inhibitor), SQ-29,548 (TP antagonist) and removal of the endothelium all abolished the vasoconstriction. These findings indicate that TxA_2 mediates the endothelium-dependent contractions in rabbit pulmonary artery (30). Furthermore, in migraine headache patients, the plasma level of 11-dehydrothromboxane B_2, a reliable indicator of TxA_2, is significantly greater than levels in tension-type headache patients or in control group patients (127). Based upon studies of the relationship between TxA_2 and certain cardiovascular diseases, TxS inhibitors and TP antagonists become potential therapies for these cardiovascular diseases (66, 75, 114, 198).

2.2.3.4.2. Prostacyclin

Vascular endothelial cells as well as VSM cells can convert PGH_2 to PGI_2 via the enzyme prostacyclin synthase. The endothelium has a much higher capacity (about 10-fold) to produce PGI_2 than VSM due to the higher COX content of the former cell type

(55, 161). PGI_2 is very unstable and spontaneously degrades to its stable metabolite 6-keto-$PGF_{1\alpha}$. The functions of PGI_2 are opposite to those of TxA_2. PGI_2 interacts with the prostacyclin receptor (IP) to cause vascular relaxation and inhibit platelet aggregation by increasing cAMP levels in platelets and VSM cells (176).

PGI_2 plays an important role in regulating cardiovascular functions. An imbalance of TxA_2 and PGI_2 is associated with the pathogenesis of some cardiovascular diseases such as pulmonary hypertension and atherosclerosis (37, 76, 106, 244). Further, increased 24-hour urinary excretion of TxB_2 and decreased 24-hour urinary excretion of 6-keto-$PGF_{1\alpha}$ are observed in postmenopausal women who have increased incidences of cardiovascular diseases (109). PGI_2 analogues are successfully used in the treatment of pulmonary hypertension (80, 183).

2.2.3.4.3. Other prostaglandins

Prostaglandin D_2 has a lower potency than PGI_2 to inhibit platelet aggregation, increase cAMP level and cause peripheral vasodilation. Another difference from PGI_2 is that PGD_2 can also cause pulmonary vasoconstriction and bronchoconstriction (42, 257).

Prostaglandin E_2 has diverse effects. There are at least four G-protein-linked subtypes of the prostaglandin E_2 receptor (EP): EP_1, EP_2, EP_3, and EP_4. EP_3 and EP_4 receptors are widely distributed throughout the body, whereas the EP_1 receptor is expressed mainly in kidney, lung and stomach, and is the least abundant among the EP receptors (26, 175). EP_2 and EP_4 receptors increase cAMP level in VSM cells via Gs protein and cause vasodilation (42). EP_1 and EP_3 receptors cause VSM contraction. The

EP_1 receptor increases calcium mobilization and the EP_3 receptor inhibits cAMP via G_i protein (42). PGE_2 was also reported to antagonize VP-induced water reabsorption in the renal collecting duct by inhibiting VP-stimulated accumulation of cAMP (77).

2.2.4. Interactions among vasoactive substances

The maintenance of vascular homeostasis requires a balance between vasodilator and vasoconstrictor functions, as well between platelet aggregation and anti-aggregation systems. Thus, there must be mechanisms present to control these opposing vascular functions. One of the mechanisms may involve the interactions among the vasoactive substances themselves. For example, NO is a major vasodilator and it can also influence the production of other vasoactive substances. Davidge et al. used a bovine coronary microvessel endothelial cell line to show that NO increased the production of both PGI_2 and TxA_2 by activation of COX (50). Peroxynitrite activates the COX activities of prostaglandin endoperoxide synthases by serving as a substrate for the enzymes' peroxidase activities (138). Peroxynitrite is the metabolite of NO; thus, it may link NO with the biosynthesis of PGs. On the other hand, Cytokine-induced iNOS expression and NO production were significantly augmented in cultured VSM cells obtained from TxA_2 receptor deficient mice (TP-/-) compared to wild-type VSM cells, indicating an inhibitory effect of endogenous TXA_2 on iNOS expression (265). Conversely, several vasoconstrictors, such as ANG II, VP, and NE, can stimulate the release of NO (as discussed above).

The release of AA and PGs can be regulated by other vasoactive substances besides NO. Kato T. et al. reported that Ach stimulated the release of endothelium-derived contracting factors in Spontaneous Hypertensive Rats (SHR) and normotensive Wistar-Kyoto (WKY) rats, and PGH_2 was a strong candidate among the various possible PGs (119). Using [^{3}H]arachidonic acid-labeled A-10 VSM cells, Lehman JJ et al. showed that VP activated the release of AA via a calcium-independent phospholipase A_2 pathway (144). In rat-1 fibroblasts, phenylephrine acted on the α-adrenergic receptor to stimulate the release of AA via activation of phospholipase D (204). In cultured rat VSM cells, Ohnaka K. et al. showed that ANG II increased COX-2 mRNA and protein expression and stimulated PGE_2 production through the mitogen-activated protein kinase (MAPK)-mediated signaling pathway (178). Thus, some cardiovascular diseases that involve elevations in ANG II, VP or other agents, may also involve enhanced release of PGs.

2.3. Sexual dimorphism in vascular function

Many studies have described marked differences between male and female cardiovascular systems. First, as described above, the incidences of many cardiovascular diseases differ between men and women (25, 73, 194). Similar sexual dimorphism even exists under normal conditions. For example, some studies using 24-hour ambulatory blood pressure monitoring have shown that in normotensive 20-79 year old people, men had higher blood pressure than age-matched women until the age of 70 to 79 (125, 261). Comparison of 100 hypertensive men and 100 age-matched hypertensive women

revealed that women had higher resting heart rate, cardiac index, and pulse pressure and lower total peripheral resistance than men with the same level of arterial pressure (158).

Second, the regulation of blood pressure differs between the sexes. The baroreflex, which reflects the minute-to-minute autonomic control of cardiovascular system, exhibits sex differences in function. Peckerman A. et al. showed that among normotensive subjects, men had a greater baroreceptor reflex sensitivity than women. In hypertensive subjects, baroreceptor reflex sensitivity was reduced in men, but not in women (188). Thus, men can respond to changes in blood pressure more quickly and accurately than can women under normal conditions, but during the development of hypertension, this mechanism is somehow altered in men, but less so in women.

The production of endothelium-derived vasoactive factors is also different. The basal release of NO from rabbit aortic rings is greater in female than in male (91). Similarly, in Wistar rats, basal and ACh-evoked release of NO is also greater in female than in male (121). Central administration of NE and ANG II stimulates greater release of VP in female than in male rats (231, 232). The plasma pool of AA is 75 percent higher in men than in women, but the turnover rate of AA, calculated per kg body weight, is significantly higher in women (89). Basal release of both PGE_2 and TxB_2 from peritoneal macrophages are similar in adult male and female Fisher 344 rats, but zymosan and calcium ionophore A23187, which are involved in the immune response, stimulated more PGE_2 and TxB_2 release in male rats (58).

Third, vascular reactivity to vasoactive agents differs between the sexes, and the differences are species-, vasculature- and agonist-specific. In the isolated, perfused rat

lung, the pressor response to the TxA_2 mimic U-46619, but not to ANG II, is greater in female than in male rats (65). In contrast, in the dog, coronary and renal vascular responses to U-46619 are greater in male than in female (117). Maximal contractile responses to KCl and the calcium channel agonist Bay K 8644 are similar in male and female rat aorta; however, the sensitivity to both KCl and Bay K 8644 are greater in the male (63). Contractile responses of the female rat aorta to VP were more than twice that of the male aorta, while the responses to PE in the female aorta were half that of the male aorta (224). Altura BM. studied the mesenteric artery in situ in intact male, female and male rats pretreated with a single dose of 17β-estradiol (2 or 10 mug/100 g s.c.) to test the influence of sex on the vascular responses to vasoactive hormones. They concluded that the dose-response curves for the constrictor catecholamines (epinephrine and NE) and neurohypophyseal hormones (vasopressin, oxytocin, vasotocin) were shifted leftward significantly in female rats compared to male. Pretreatment of male rats with estrogen resulted in an enhancement of the constrictor actions of the catecholamines and the neurohypophyseal hormones (5). In Spontaneously Hypertensive Rat (SHR) aorta, ACh-induced endothelium-dependent relaxation is more pronounced in female, but ACh only evokes endothelium-dependent contraction in quiescent thoracic aortic rings from male but not from female SHR (121). These sex differences in vascular reactivity may result from differences in agonist-induced downstream cellular events in female and male, from sex differences in agonist-stimulated release of endothelium-derived vasoactive substances, or from male-female differences in expression and sensitivity of receptors to those agonists. For example, Cunard CM. et al. showed that

the maximal response of rat pulmonary artery to U-46619 and NE were not different between female and male, but after removal of the endothelium, the response to U-46619 was decreased only in female and the response to NE was not affected by denuding the endothelium (48). This experiment suggests that U-46619 but not NE may stimulate the release of an endothelium-derived contractile factor in the female rat pulmonary artery.

The sexual dimorphism observed in cardiovascular system functions strongly suggests that sex steroid hormones play an important role in the regulation of the cardiovascular system.

2.4. Effects of estrogen on the cardiovascular system

2.4.1. Metabolism of estrogen

The female sex steroid hormones include estrogen (E_2: estradiol and E_1: estrone) and progesterone. They are mainly produced in the ovary from cholesterol and, particularly in postmenopausal women, from conversion of androgen precursors in peripheral tissues (especially adipose tissue). The biosynthesis of estrogen is shown in Fig. 5.

There are multiple pathways for estrogen metabolism in the liver as well as estrogen-responsive target organs (Fig. 6). These include oxidative metabolism by NADPH-dependent cytochrome P450 enzymes (hydroxylations) (153), and conjugative metabolism by glucuronidation, sulfation (93, 270) and O-methylation (11). Metabolites of estrogen are often inactive, but some do possess biological activity. The metabolites exert their biological effects either by the classical cytoplasmic estrogen receptors or by

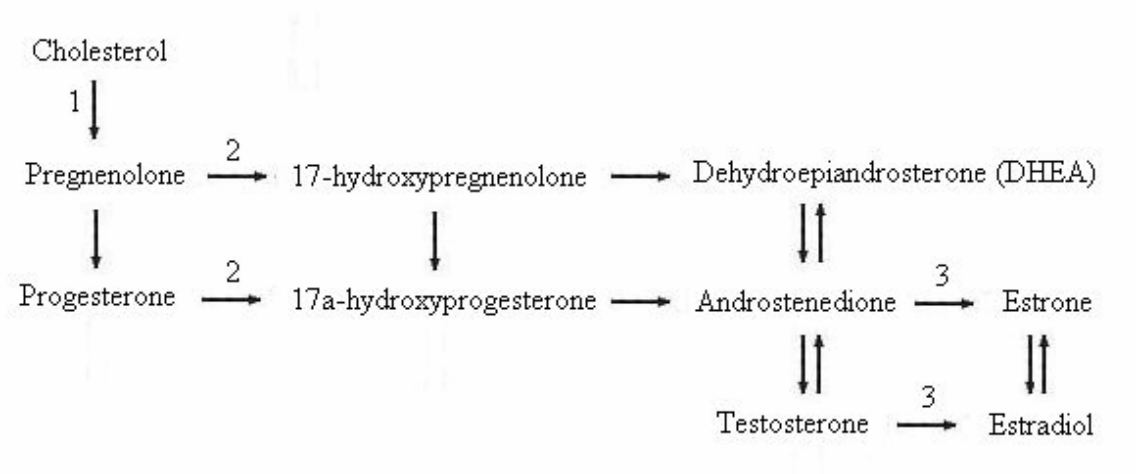

Fig. 5. Biosynthesis of steroids in the ovary. 1, Cholesterol side chain cleavage enzyme complex; 2, 17α-hydroxylase; 3, aromatase.

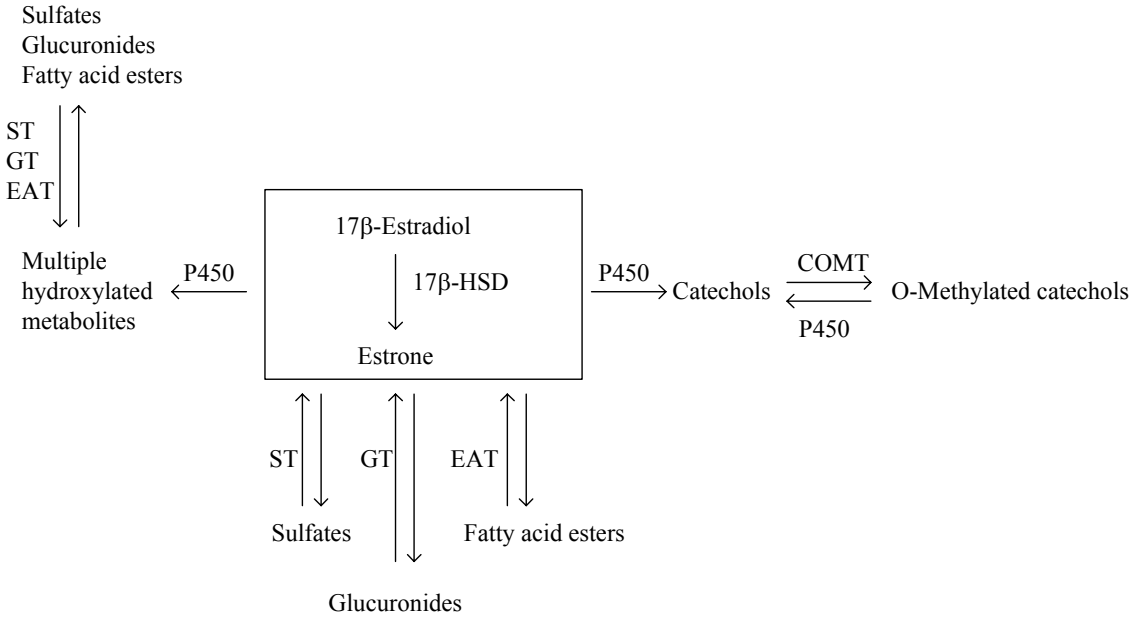

Fig. 6. Multiple pathways for estrogen metabolism. ST, sulfotransferase; GT, glucuronosyltransferase; COMT, Catechol-O-Methyltransferase; 17β-HSD, 17 β-hydroxysteroid dehydrogenase. EAT, estradiol acyltransferase.

different membrane-bound receptors (270). Estradiol and estrone are both converted into the metabolite estriol in the liver. Estrogenic hormones are eliminated from the body by the kidney as water-soluble metabolites, such as glucuronides and sulfate, or as lipid-soluble metabolites, such as estriol, via urinary excretion.

2.4.2. Estrogen receptors

The estrogen receptor (ER) belongs to the nuclear receptor superfamily. In 1966, Toft and Gorski identified a protein in the rat uterus that specifically bound to ^{3}H-17β-estradiol (239). Human ER was first cloned and sequenced from MCF-7 human breast cancer cells (86). In 1996, Kuiper et al. cloned a novel estrogen receptor from a rat prostate cDNA library (135). This subtype of ER is named ER_{β} and the first ER is named ER_{α}.

2.4.2.1. The structure of ERs

The ER contains six different functional domains (Fig. 7). Starting from the N-terminal end is the A/B domain, which contains 185 amino acids and is the least conserved region of the ER. It contains a constitutive transcriptional activation function 1 (AF-1). AF-1 is tissue- and cell-specific, hormone-independent, and is involved in protein-protein interactions with other transcription factors (242). C domain has 66 amino acids, contains the DNA binding domain DBD, and is responsible for recognition of specific DNA sequences and DNA binding. The DBD in the ER has two zinc finger-like structures. The first zinc finger directly interacts with the estrogen response element

(ERE) in the target gene promoter region, which is a specific 13-bp palindromic sequence in the DNA major groove, and is responsible for the recognition of this sequence (212, 213). The second zinc finger is involved in the formation of ER homodimer. The D region of ER contains the nuclear location signal and is also involved in protein-protein interactions (267). The E region of the ER is a complex domain that has several important functions, including association with heat shock proteins, ligand binding, dimerization, nuclear translocation, and transcriptional activation function 2 (AF-2). AF-2 is ligand-dependent and can act independently or synergistically with AF-1 to activate transcription. The differential activities of AF-1 and AF-2 are dependent on promoter- and cell- context, and may be mediated by adaptors or coactivators that are specific to one or the other domain. The function of the F region of the ER is unclear; it is not well conserved, and it may affect the agonist/antagonist effects of antiestrogens (164).

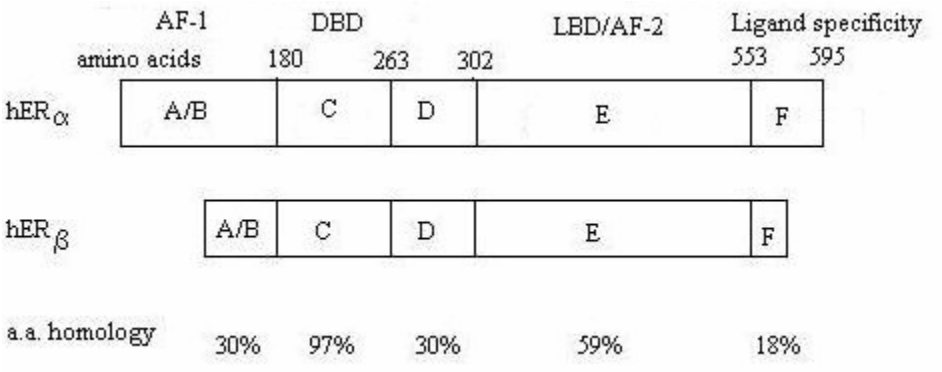

Fig. 7. Structure and homology of human ER_α and ER_β proteins.

2.4.2.2. ER_β

In 1996, Kuiper et al. cloned a novel estrogen receptor, ER_β, from a rat prostate cDNA library (135). Subsequently, human and mouse ER_β was cloned (167, 241). ER_α is on human chromosome 6, whereas, ER_β is on chromosome 14 (64). ER_β is highly homologous to ER_α (Fig. 7); however, the AF-1 region in the A/B domain and the F region in ER_α and ER_β are different, suggesting that their hormone-independent activity and protein interactions may be different.

The tissue distribution and relative levels of ER_α and ER_β are different. ER_α is expressed in most female organs including the ovaries, the reproductive tract, the mammary glands, the cardiovascular system and the brain. In contrast, ER_β is highly expressed in the ovary, but is present in lower amount in other tissues. However, it was reported that ER_β is the ER form that predominantly expressed in human vascular smooth muscle cells, especially in women (99). ER_β is also expressed in male organs and different areas of the central nervous system. In the mouse, ER_β is highly expressed in prostate and ovaries, but low levels are observed in the uterus, lung, testis, brain and artery (45).

ER_α and ER_β may signal in different ways depending upon the ligand and response element. For example, at the AP1 site, 17β-estradiol activates transcription with ER_α, but inhibits transcription with ER_β. The antiestrogens, like tamoxifen, raloxifene, and ICI 164384 are potent transcriptional activators with ER_β at the AP1 site (184).

2.4.3. Mechanisms of ER-mediated long-term (genomic) action

2.4.3.1. Hormone dependent signaling pathway

In the absence of ligand, ER is associated with heat shock proteins (hsp90, hsp70 and hsp56) (16, 192) and remains inactivated. Ligand binding phosphorylates several sites of the ER and results in a conformational change, heat shock protein disassociation, receptor homodimerization, and binding of the receptor with a specific region of the DNA (estrogen response element, ERE), which then stimulates gene transcription (Fig. 8, pathway 1). The second pathway for ER-mediated gene transcription is dependent

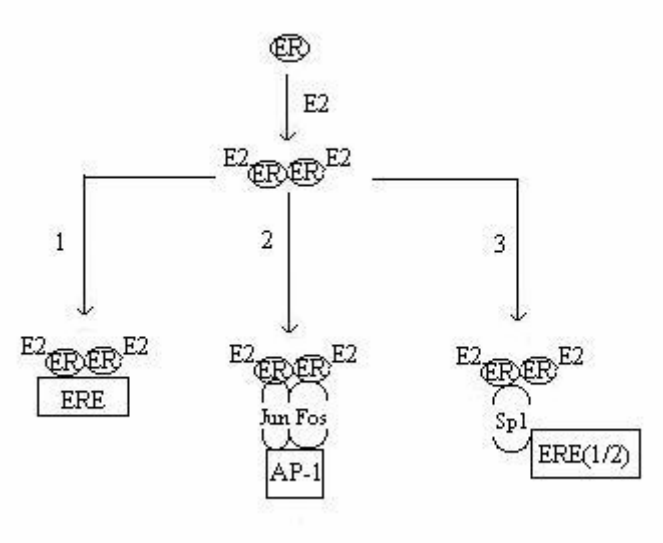

Fig. 8. Models of ER-mediated transactivation.

upon the AP1 site in the promoter. In this pathway, ER binds to Fos-Jun proteins that bind to the AP1 enhancer motif (233) (Fig. 8, pathway 2). Some EREs only have half-site palindromes. In this situation, the half-site ERE motif is stabilized by selective

transcription factor 1 (Sp1), and ER does not interact with ERE directly, rather it interacts with the Sp1/ERE(1/2) complex to stimulate gene transcription (191) (Fig. 8, pathway 3).

2.4.3.2. Hormone-independent ER-mediated transactivation

Many studies have shown that growth factors can phosphorylate ER, specifically in the A/B region, via mitogen-activated protein kinase (MARK), and thus activate ER and induce ER-mediated gene transcription (8, 28).

2.4.4. Membrane ER and short-term (non-genomic) action of estrogen

In addition to the classic cytoplasmic ER, several studies have reported the existence of a plasma membrane ER (mER) (20). Although linkage to the signaling pathways inside cell is not well understood, many studies have shown that mER has multiple functions. Within a few seconds to minutes after binding with mER, estradiol can trigger an intracellular Ca^{2+} spike through a PLC-dependent mechanism involving store-operated Ca^{2+} channels (190) and stimulate a PKC pathway (140). Activation of mER can also stimulate rapid NO release and cGMP formation following a transient increase in intracellular Ca^{2+} level (206, 228). Very low concentrations of estrogen (pM ~ nM) can stimulate rapid cAMP generation (9). Estrogen can also stimulate rapid opening of Ca^{2+}-activated K^+ channels via NO and cGMP, and both NO and K^+ channel activation will lead to vasodilation (203). Estrogen acts upon plasma membrane ER that is located in membrane caveolae and directly activates eNOS and stimulates NO release

without changing cytosolic Ca^{2+} concentration (126). mER can also regulate transcription; for example, mER can activate fos transcription via extracellular-signal-regulated kinase (ERK) in a human neuroblastoma cell line SK-N-SH (258).

2.4.5. Effects of estrogen on the cardiovascular system – the controversy

ERs are expressed throughout the cardiovascular system and they are present in both vascular endothelial cells (7, 38, 41) and VSM cells (7, 103, 173, 181, 196). Treatment of the female baboon and the male rat with 17β-estradiol causes reductions in ER number in cytoplasmic preparations and enhancement of ER in nuclear preparations from both aorta and myocardium of both species (146, 147). The ability of 17β-estradiol to alter localization of cardiovascular estrogen receptors between cytoplasmic and nuclear fractions suggests that these ERs are physiologically functional and indicates that estrogen may directly regulate cardiovascular cell function. Estrogen exerts long-term, genomic effects on the vascular system by altering expression of genes that regulate synthesis of proteins that influence vascular tone and homeostasis. Estrogen also exerts non-genomic effects that rapidly induce vasodilation. The effects of estrogen on the cardiovascular system appear to involve both protective and deleterious effects.

2.4.5.1. The protective effects of estrogen

Epidemiological studies reveal that prior to menopause, the occurrence of some cardiovascular diseases is significantly lower in women than in men, but after menopause, the incidences of cardiovascular diseases in women increases to levels

similar to those in men. These data suggest that female gonadal steroid hormones have protective effects on the cardiovascular system. Many experiments have investigated these effects of estrogen on the cardiovascular system and the underlying mechanisms, and most have reported that estrogen does exert protective effects on the cardiovascular system (Fig. 9).

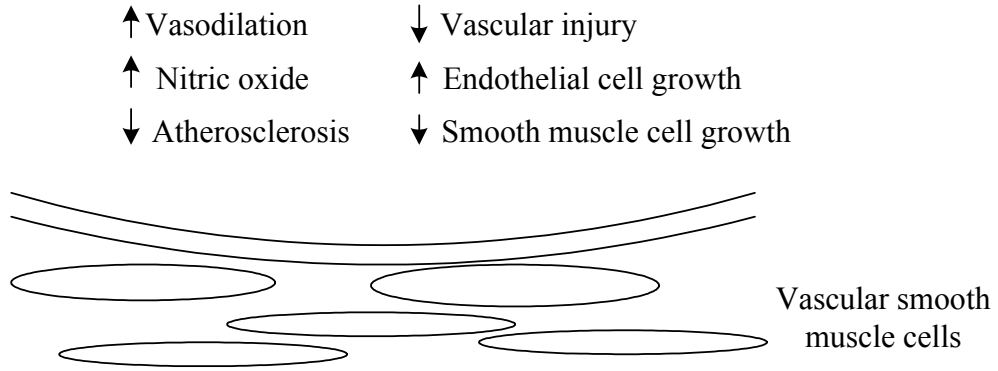

Fig. 9. Direct effects of estrogen on blood vessels.

2.4.5.1.1. Estrogen and anti-atherosclerosis

Estrogen prevents the development of atherosclerosis through its effects on serum lipid metabolism, coagulation, and fibrinolysis proteins. Oral and transdermally administered estrogen-progestin combinations reduce serum total and LDL cholesterol and triglycerides in postmenopausal women (260). In postmenopausal women undergoing diagnostic coronary angiography, estrogen treatment significantly increases mean high-density lipoprotein (HDL) cholesterol level and decreases mean total/HDL cholesterol ratio, compared to control women who did not receive estrogen therapy (100).

The effects of estrogen on coagulation and fibrinolytic cascades are shown in Fig. 10. Estrogen has both prothrombotic and antithrombotic effects. It can increase factors VII and factor Va, and decrease antithrombin III and protein C, which favor the formation of thrombosis; conversely, it also increases plasminogen, decreases fibrinogen and plasminogen activator inhibitor-1, which prevents the formation of thrombosis. The net effect is unclear. Some studies indicate that estrogen increases the release of TxA_2 to cause platelet aggregation (262). The prothrombotic action and the effect to increase TxA_2 formation may be responsible for the increased risk for venous thrombosis that occurs with the use of oral contraceptives and postmenopausal hormone replacement therapy (49).

2.4.5.1.2. Estrogen and vascular injury

The protective effect of estrogen against vascular injury appears to be mediated by its proliferative effect on endothelial cells and antiproliferative effect on VSM.

17β-estradiol enhances human umbilical vein endothelial cell proliferation, both in vitro and in vivo (165). Estrogen also inhibits apoptosis in human umbilical vein endothelial cells in a dose-dependent, receptor-mediated manner (222). The proliferative effect of estrogen on the endothelium may be due in part to its effect to increase expression of vascular endothelial growth factor (VEGF) (133). Estrogen inhibits the migration and proliferation of VSM cells (21, 130). 17β-Estradiol inhibits uptake of [^{3}H]thymidine by female rats, an index of cell proliferation; furthermore, preincubation with tamoxifen, an ER antagonist, abolishes this inhibitory effect of estradiol, which

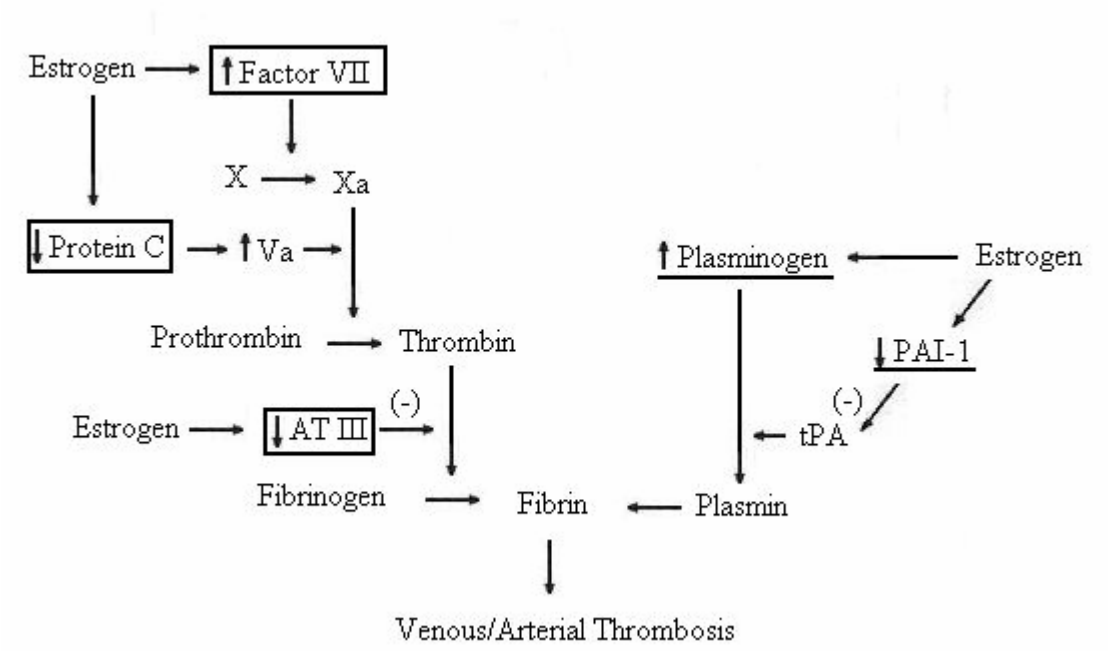

Fig. 10. Effects of estrogen on the coagulation and fibrinolytic cascades. Up or down arrows indicate effects of estrogen. The pro-thrombotic effects of estrogen are boxed; the anti-thrombotic effects are underlined. (-) indicates inhibitory action. ATIII, antithrombin III; PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator.

suggests that estrogen-induced inhibition of proliferation of rat carotid artery segments is mediated through activation of estrogen receptors (246). After balloon injury of the rat aorta, ER_α mRNA was not changed in the male, but ER_β mRNA increased in both endothelium and luminal VSM cells (148). These data suggest that ER_β mediates the protective effects of estrogen on vascular injury.

2.4.5.1.3. Estrogen and vascular reactivity

NO is probably the most important local vasodilator. Estrogen can induce the release of NO, and thereby regulate vascular tone. Treatment of guinea pigs with

estradiol increases mRNA levels for both eNOS and nNOS in skeletal muscle vasculature (259). Exposure of cultured human aortic endothelial cells to 17β-estradiol enhances Ca^{2+}-dependent NO release and increases NOS protein production (98). Further, 17β-estradiol attenuates PE-induced vasoconstriction of the endothelium-denuded rat aorta through induction of iNOS in non-endothelial cells (22). In addition to its genomic effects on NOS gene expression, estrogen can also induce a rapid release of NO, either by activating eNOS (126) or by transiently increasing intracellular Ca^{2+} concentration (98). EM-652 (acolbifene), a fourth-generation selective ER modulator (SERM), not only increases eNOS protein levels during prolonged treatments, but also triggers NO release by human umbilical vein endothelial cells through nongenomic mechanisms, rapidly activating eNOS via an ER-dependent sequential activation of MAPKs and PI3K/Akt pathways (218).

In the female rabbit, 17β-estradiol treatment induces relaxation of both the intact aorta and the coronary arteries in the perfused heart, coincident with NO formation. However, after removal of the endothelium, 17β-estradiol also causes direct relaxation of aortic and coronary smooth muscle, independent of NO formation (151). The direct effect of estrogen on VSM cells appears to occur via inhibition of Ca^{2+} entry from L-type Ca^{2+} channels (43). Using the whole-cell voltage clamp technique, Nakajima T. et al. showed that estrogen might play a role in regulating vascular tone by selectively inhibiting voltage-dependent L-type Ca^{2+} currents in cultured rat thoracic aortic smooth muscle cell lines (A7r5) (172).

Estrogen also stimulates the release of vasodilator prostaglandins. For example, estrogen increases the release of 6-Keto-$PGF_{1\alpha}$ by aortic rings obtained from male Sprague-Dawley rats, whereas, testosterone does not. Interestingly, western blots revealed that preincubation of male rat aorta with estrogen decreases COX-1 and prostacyclin synthase protein production (171). This study suggests that estrogen can stimulate the release of PGI_2 from male rat aortic rings through a mechanism not involving COX-1 or prostacyclin synthase.

Estrogen also has effects on the renin-angiotensin system (RAS). Long-term treatment with estrogen decreases angiotensin-converting enzyme (193) and down-regulates both AT_1 receptor mRNA expression and receptor density in ovariectomized female rat aorta (177); however, opposite effects of estrogen on the RAS were also reported. For example, oral estrogen replacement theraphy increases angiotensinogen levels (211). Similarly, long-term treatment with Premarin (conjugated equine estrogens) significantly increased circulating renin and ANG I levels in OvX monkeys (27). The overall effect of estrogen on the RAS is unclear and is under continued examination.

2.4.5.2. The deleterious effects of estrogen

Although estrogen appears to exert protective effects on the cardiovascular system, hormone replacement therapy (HRT) does not produce significant beneficial effects in postmenopausal women (94, 95, 105). Some studies have even shown an increased risk for cardiovascular events in the first year, including venous thrombosis. These observations may be explained by the older age and established coronary heart

disease of the participants, the combined use of estrogen with an androgenic progestin (medroxyprogesterone), or by the possibility that estrogen may also have deleterious effects on the cardiovascular system.

Several studies have shown that estrogen increases contractile responses to vasoconstrictor agonists. For example, Rorie et al. showed that estrogen (100 μg/day for 4 days) increased vascular reactivity of isolated rabbit saphenous veins to NE, as well as methoxamine and PE (202). Similarly, contractile responses of rat thoracic aorta to VP are three-fold greater in female than in male, and indomethacin significantly attenuates the contractile responses in female but not in male aorta (70, 224). This study suggests that ovarian steroid hormones may enhance aortic contractile responses of the female aorta to VP through COX products of AA. Similarly, treatment of ovariectomized rabbits with 17β-estradiol enhances the contractile responses of endothelium-intact, but not -denuded, aortic rings to NE. Likewise, indomethacin inhibits contractions to NE in endothelium-intact aortic rings from estrogen-treated rabbits (159). These results suggest that chronic treatment with estrogen can enhance arterial reactivity to NE through an endothelium-dependent mechanism that may involve COX metabolites of AA.

Studies on the effects of estrogen on the vasculature not only demonstrate increased reactivity to VP and NE, but also suggest that the release of and response to locally-produced constrictor prostanoids are enhanced in the systemic vasculature. For example, Witter et al. showed that estrogen increased the release of TxA_2 from cultured umbilical cord endothelial cells (262). In the isolated, perfused rat lung, pressor responses to TxA_2 mimic U-46619 are greater in female than in male rats, and estrogen

enhances the responses to U-46619 (65). Further, indomethacin attenuated contractile response of female rat aorta to VP, while ovariectomy attenuated contractile response to VP but also abolished the attenuating effects of indomethacin (70).

The increased reactivity of the female vasculature to constrictor prostanoids may result from the increased expression and/or activity of enzymes such as COX and/or TxS that mediate formation of these local vasoconstrictors, and/or from the increased expression of the TP. For example, indomethacin attenuated maximal responses to VP in female but not male rat aortas, and SQ-29,548 (a TP antagonist) had a virtually identical effect as indomethacin in the female rat aorta (70). Further, indomethacin, the COX-2-selective inhibitor nimesulide, and SQ-29,548, all significantly reduced maximal contractions of the endothelium-denuded rat aorta to the α_2-adrenoceptor agonist clonidine (partial agonist relative to phenylephrine), compared to control (44). The controversial observations summarized above emphasize the need to further elucidate the effects of estrogen on vascular function and the underlying mechanisms.

2.4.6. Phytoestrogens

Phytoestrogen is a general term used to define plant-derived, estrogen-like molecules that include the isoflavones (genistein, daidzein), coumestans, and lignans. Beans and legumes are abundant sources of isoflavones, and lignans are widely found in cereals, fruits and vegetables (170). In humans, phytoestrogens are converted in the gastrointestinal tract to heterocyclic phenols that are structurally similar to estrogen (Fig. 11).

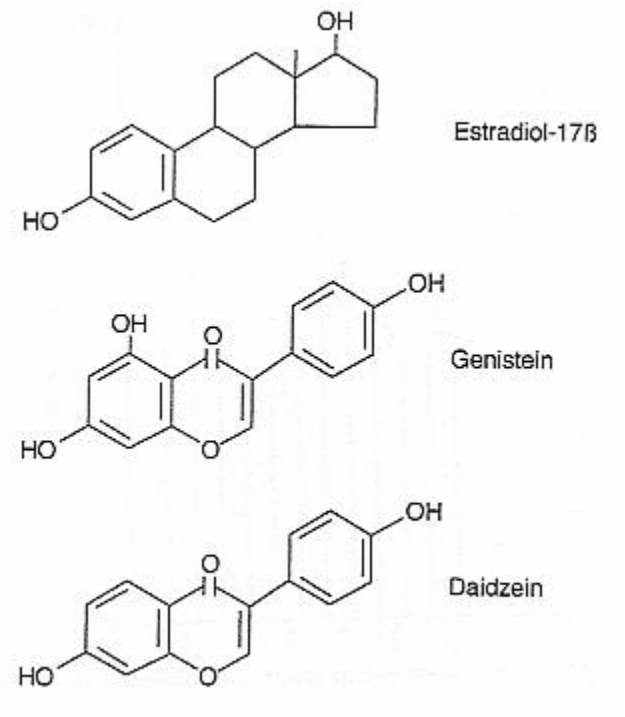

Fig. 11. Comparison of the molecular structures of 17β-estradiol and phytoestrogen (genistein and daidzein).

Phytoestrogens appear to interact selectively with ER_β over ER_α, although they have a relatively low affinity for both receptors. Compared to estradiol, the binding affinity of genistein for ER_α is 4%, and for ER_β is 87%; similarly, the binding affinity of daiazein for ER_α is 0.1%, and for ER_β is 0.5% (135). The biological activities of phytoestrogens have been determined mainly in animal studies. Phytoestrogens have both estrogenic and antiestrogenic effects, depending upon the specific tissue and the concentrations of circulating endogenous estrogens. For example, in the human ER-positive MCF-7 breast cancer cell line, genistein stimulates cell growth at low concentrations [10^{-5}–10^{-8} M]; but at higher concentration [10^{-4}–10^{-5} M], it exerts

antiestrogenic effects and inhibits cell growth (256). Phytoestrogens also exert estrogenic effects on lipid metabolism (237) and inhibit growth of VSM cells (59) that may contribute to their cardiovascular protective effects observed in Asian countries and in vegetarians (2, 40). The effects of phytoestrogens on vascular reactivity are unclear. A study on rhesus monkeys with pre-existing diet-induced atherosclerosis showed that coronary arteries from male constricted in response to Ach, and that arteries from female in the low-isoflavone group constricted in response to ACh, whereas arteries from female in the high-isoflavone group dilated. Intravenous administration of genistein caused dilation in low-isoflavone female that previously exhibited vasoconstrictor response to ACh. It was concluded that dietary soy isoflavones enhanced the dilator responses to ACh in atherosclerotic arteries in female monkeys (101). The effects of phytoestrogens on the vascular system still remain uncertain, and need to be elucidated in much further detail.

CHAPTER III

EXPERIMENTAL DESIGN AND METHODS

3.1. Major objectives and central hypothesis

Previous studies reveal that male-female differences exist in normal vascular function and in the occurrence of some human vascular diseases. Estrogen may enhance the constrictor prostanoid pathway and/or expression of TP to increase vascular tone, and thereby, may contribute to sexual dimorphism in vascular function and vascular disease. Therefore, the major objectives of this doctoral dissertation are: 1) To determine male-female differences in constrictor prostanoid function and vascular contraction in the rat aorta; and 2) To elucidate the role of estrogen in the regulation of constrictor prostanoid function in the rat aorta.

The central hypothesis to be tested is that: estrogen potentiates agonist-induced vascular contraction of the female rat aorta by upregulating the production of constrictor prostanoids (TxA_2 and/or PGH_2) and/or TP expression.

This hypothesis will be tested by addressing the following specific aims:

1) To determine male-female differences in constrictor prostanoid function and vascular contraction in the rat aorta in vitro, and the role of estrogen in the regulation of these mechanisms.
2) To determine male-female differences in TxA_2 and PGI_2 release by the rat aorta in vitro and the role of estrogen in the regulation of the TxA_2 and PGI_2 biosynthesis pathway.

3) To determine the molecular mechanism(s) by which estrogen upregulates constrictor prostanoid function in the rat aorta.

3.2. Materials and methods

3.2.1. Animal/tissue preparations

3.2.1.1. Animals

Age-matched (14-18 weeks old) female and male Sprague-Dawley rats (Harlan Labs. Inc.) were used in all studies. The rats were housed in vivarium facilities at the College of Veterinary Medicine (Laboratory Animal Resources and Research facility; LARR) with controlled temperature (22-24 °C), relative humidity (approx. 50%) and lighting cycle (12:12 L/D). The animals were segregated by sex and housed in pairs in standard plastic laboratory rat cages. Tap water and either standard laboratory chow (22% protein) or a phytoestrogen-free diet (soy and/or alfalfa were replaced with casein as the major source of protein) (7% Corn Oil Diet; Harlan, Inc.) was provided ad libitum according to the experiments.

3.2.1.2. Animal treatment regimens

Animals were randomly separated into 4 experimental groups: male, intact (InT)-female, ovariectomized (OvX)-female, and OvX+estrogen-replaced (OvX+Est)-female. The estrous cycle of the InT-female rat was ignored since previous studies have shown that neither the contractile responses of the female rat aorta to VP or PE, nor stimulation of prostaglandin production by catechol estrogens differ with phase of the estrous cycle

(124, 224). For each group, sample sizes of 3-9 rats were used to account for experimental loss and known levels of statistical variability in vascular experiments and molecular studies in vitro. Ovariectomy surgery was performed when female rats were 4-5 weeks old, using standard methods, and estrogen was replaced at the age of 8-9 weeks for 21-28 days by subcutaneous implant of 2-0.05 mg, 60-day-release pellets of 17β-estradiol (Innovative Research, Inc.; Sarasota, FL). Previous studies have shown that this dose produces physiological concentrations of 17β-estradiol in the plasma of female rats (250).

3.2.1.3. Preparation of isolated thoracic aorta

After sacrifice by rapid decapitation, the thoracic aorta was gently removed to avoid stretching the vascular smooth muscle or damaging the endothelium. The aorta was then placed in chilled Krebs-Henseleit-bicarbonate solution (KHB) (4° C) and kept gassed with 95% O_2/5% CO_2. The composition of the KHB included (in mM) 4.74 KCl, 2.5 $CaCl_2$, 25.0 $NaHCO_3$, 118.0 NaCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, and 10.0 Glucose. All adipose and connective tissue were removed and the mid-thoracic region was sectioned into rings (3 mm length). Aortas were studied either with the endothelium intact (Endo+) or with the endothelium denuded (Endo-) by gently passing a frayed nylon string through the lumen (225). The rings were then carefully mounted on two 25-gauge stainless steel wires. The upper one was attached to a force-displacement transducer (Grass FT-03D), and the lower one was attached to a stationary stainless steel rod and micrometer, which allowed adjustment of the passive tension of the ring (224).

The transducer was connected to a polygraph (Gould 2600S) for a continuous record of the isometric tension produced by the aortic ring.

3.2.2. General experiment methods

3.2.2.1. Isometric tension studies

Immediately after mounting, aortic rings were immersed in water-jacketed organ baths filled with 15.0 ml warmed (37 °C) KHB solution continuously gassed with 95% O_2/5% CO_2. Passive tension was then gradually (over a 30 minute period) increased to 2.50 g, which is optimal for both male and female rat aortas, as demonstrated in previous studies (224, 225). The tissues were allowed to equilibrate for 90 minutes, and the KHB was replaced with warmed, freshly gassed KHB solution every 20 minutes, and passive tension was adjusted to maintain 2.50 g. After equilibration, a near-maximal concentration of PE ($1x10^{-6}$ M, final concentration) was added to the baths to stabilize the aortas; after the contractions attained a stable plateau tension, the endothelium-dependent vasodilator acetylcholine (ACh, $1x10^{-7}$ M) was added to the baths to test the functional integrity of the endothelium. After the maximal relaxation was attained, the baths were changed twice and the tissues were allowed to re-equilibrate for 30-45 minutes. A second stabilizing contraction and relaxation to PE and ACh were then obtained as described above. After a second re-equilibration period, either U-46619 (1×10^{-11} - 3×10^{-6} M) or VP ($1x10^{-11}$ - $1x10^{-6}$ M) was added to the baths in a cumulative manner to obtain a concentration-response curve for each ring. At each concentration, a stable plateau tension was attained before increasing the concentration of the agonist.

3.2.2.2. Basal and agonist-induced release of TxB_2 and 6-keto-$PGF_{1\alpha}$

Paired, adjacent aortic rings (3 mm length) obtained from each of the four experimental animal groups, were cleaned of all connective tissue and fat, and were then placed into chilled (4 °C), KHB solution, gassed with 95% O_2/5% CO_2, and allowed to stabilize for at least 30 minutes. The paired rings were then transferred into 12x75 mm plastic culture tubes containing 2 ml chilled KHB, and allowed to gradually warm to 37 °C. The tissues were then pre-incubated at 37 °C and gassed continuously for 30 minutes, and the KHB solution was then carefully aspirated. Immediately thereafter, 1.0 ml of either KHB alone (basal) or KHB with low-dose (10^{-8} M) VP or high-dose (10^{-6} M) VP was added to the tubes and the tissues were incubated for 45 minutes at 37 °C and gassed constantly with 95% O_2/5% CO_2. After incubation, the KHB was aspirated and stored at –70 °C until radioimmunoassay (RIA) of TxB_2 and 6-keto-$PGF_{1\alpha}$. For some experiments, the aortas were pre-incubated in the presence of Indo (10 μM), NS-398 (10 μM), or vehicle-control.

Concentrations of TxA_2 and PGI_2 in the incubation media were measured in each sample using specific RIA for their stable metabolites TxB_2 and 6-keto-prostaglandin $F_{1\alpha}$ (6-Keto-$PGF_{1\alpha}$), respectively. Prostanoid standards (0.975-1,000 pg for TxB_2 and 1.95-1,000 pg for 6-Keto-$PGF_{1\alpha}$) or unknown samples were incubated with [^{3}H]TxB_2 or [^{3}H]-6-keto-$PGF_{1\alpha}$ and with the appropriate prostanoid antiserum overnight for 16-20 hr at 4 °C. The charcoal-dextran method was used to separate bound and free fractions of [^{3}H]TxB_2 or [^{3}H]-6-keto-$PGF_{1\alpha}$. Bound radioactivity was counted by liquid scintillation spectroscopy. The limit of detection of the RIAs was 1.95 pg/tube for TxB_2 and 3.90

pg/tube for 6-Keto-$PGF_{1\alpha}$; the cross-reactivity of the antiserum to other prostanoids was <0.1%, and the intra-assay and interassay coefficients of variation were 5.0% and 7.6% respectively (227).

3.2.2.3. Immunohistochemistry of COX-1, COX-2 and TxS

Freshly isolated thoracic aortas were cannulated with a 16 ga. needle and gently flushed with 10% neutral buffered formalin to remove blood and air from the lumen. The open ends of aortas were then ligated with 4-0 silk suture and the whole aortas were immersed in formalin and fixed under pressure (80 mmHg) for 24 hours. After fixation, the aortas were dehydrated with graded ethanol, embedded in paraffin, and cut into 4 μm thick sections. After cleaning with xylenes and rehydration with graded ethanol, the antigens (COX-1, COX-2, or TxS) were retrieved using the microwave oven heating method. Primary antibodies against TxS, COX-1 or COX-2 (Cayman Chemical, Ann Arbor, Michigan) were then applied, and binding was detected using a biotinylated secondary antibody (Cayman Chemical; Ann Arbor, Michigan) in combination with the avidin-biotin-horseradish peroxidase method and 3,3'-diaminobenzidine (DAB, Vector Laboratories, Inc., Burlingame, CA) as a chromagen. An adjacent section was used as a negative control, and was treated only with secondary antibody. Hematoxylin was used as a counterstain (30, 88).

3.2.2.4. Reverse transcription-polymerase chain reaction (RT-PCR) for COX-1, COX-2, TxS and TP

Freshly isolated aortas were opened longitudinally and the endothelial cells were gently removed with a cotton swab that was then placed into 1.0 ml of Trizol reagent kept on ice. The remaining aorta was then scrubbed with another cotton swab to remove any remaining endothelial tissue and the resulting VSM was then placed into 1.0 ml of Trizol reagent kept on ice. Freshly isolated endothelium and VSM were then homogenized in the 1.0 ml Trizol reagent and the RNA was extracted in 200 μl chloroform, 5 μl (25 μg/ml) glycogen (Ambion), 50 μl Na acetate (5 M), 500 μl isopropyl alcohol, 1 ml 75% ethanol and centrifuged at 4 °C, according to the manufacturer's instructions. The extracted RNA was quantified by uv absorbance at 260 nm and stored at –80 °C. Equal amounts (0.2 μg) of extracted RNA from each sample were subjected to reverse transcription by reverse transcriptase (Thermoscript RT-PCR system, GibCoBrl, Life Technologies, Grand Island, NY), using 1 μl specific antisense primers (Sigma-Aldrich, Genosys) in a PCR buffer (4 μl 5x buffer, 2 μl 10 mM dNTP, 1 μl 0.1 M DTT, 1 μl RnaseOut, 1 μl Thermoscript RT) in a 20 μl total volume. The RT reaction was performed according to the manufacturer's instructions. 2 μl or 5 μl cDNA, according to the gene expression, was then amplified in a total volume of 50 μl containing 1 μl 5' specific primer, 1 μl 3' specific primer, 1 μl Taq polymerase (Expand High Fidelity, Roche Molecular Biochemical, Indianapolis, IN), 5 μl 10x PCR buffer without $MgCL_2$, 2 μl 10 mM dNTP, and 3 μl $MgCl_2$. After an initial 4-min denaturation

step at 94 °C, the TxS cDNA was amplified for 35 cycles, and the primers were annealed at 54 °C. COX-1 and COX-2 cDNA were amplified for 30 cycles and the primers were annealed at 55° C. TP cDNA was amplified for 35 cycles and the primers were annealed at 55 °C. Each cycle consisted of 30s denaturation at 94 °C, 30s annealing at the specific temperature (except primers for TxS, which were annealed for 60s because of the longer length), and extension at 72 °C for 30s, followed by 7 min final extension at 72 °C. The RT-PCR product was separated by gel electrophoresis on 1.8% agarose gel, visualized with ethidium bromide staining using Gel Doc 1000 system (Bio-Rad laboratories, Hercules, CA, USA), and quantified by densitometry (Multi-analyst + /Macintosh software, Bio-Rad laboratories). The PCR fragments were identified by size using standard DNA (ϕX174RF DNA/Hae III Fragments, Life technologies). The results were expressed as the light intensity ratio of the 'specific' mRNA and the housekeeping gene GAPDH (glyceraldehydes-3-phosphate dehydrogenase) amplified in the same tissues. The negative control was performed under the same conditions without Taq polymerase. The specific primers used are shown in Table 2. (19, 92).

3.2.2.5. Drugs

The following drugs were used: 17β-estradiol (0.05 mg 60-day-release pellets; Innovative Research of America; Sarasota, FL), U-46619 (15-hydroxy-11, 9-epoxymethanoprosta-5Z, 13E-enoic acid), NS-398 (N-[2-cyclohexyloxy-4-nitrophenyl]-methanesulfonamide) and Niflumic Acid (Cayman Chemical; Ann Arbor, Michigan), arginine vasopressin (VP, Bachem Inc., Torrance, CA), phenylephrine hydrochloride

Table 2. Specific primers (sense and antisense) for COX-1, COX-2, TxS, TP and GAPDH were used for RT-PCR.

Gene	Oligonucleotide Primers	Size of PCR Product	Reference
COX-1	Sense: 5'CTCACAGTGCGGTCCAAC3' Antisense: 5'CCAGCACCTGGTACTTAAG3'	424bp	Bernard N, 2000; Kawaguchi H, 1994.
COX-2	Sense: 5'GAAATGGCTGCAGAGTTG3' Antisense: 5'GGAATTCTCATCTAGTCTGGAGAGTTG3'	356bp	Bernard N, 2000; Jensen BL, 1997.
TxS	Sense: 5'TCCAGAGGTGTTACTGCTGT3' Antisense: 5'GAAGCATGACAAACATTTATTC3'	1,187bp	Bernard N, 2000; Tone Y, 1994.
TP	Sense: 5'TGCTGCAGACGCTACCTGTC3' Antisense: 5'GATTGGCACCGTCCTTCAGG3'	244bp	Nanji AA, 1997.
GAPDH	Sense: 5'GTGAAGGTCGTGTCAACGGATTT3' Antisense: 5'CACAGTCTTCTGAGTGGCAGTGAT3'	558bp	Bernard N, 2000; Hughes AK, 1995.

(PE), acetylcholine (ACh) and indomethacin (Indo, Sigma Chemical; St. Louis, MO), Dazoxiben HCl (DAZ, Pfizer Central Research; Sandwich, England), DAB (Vector laboratories, Inc.; Burlingame, CA), and Trizol reagent (Life Technologies; Rockville, MD, USA). Stock solutions of these drugs were prepared fresh daily except for VP

(which was diluted daily from aliquots of 1 x 10^{-3} M stock solution stored at –70 °C) and NS-398 (which was diluted daily with DMSO from 1 mg/ml stock solution stored at –20 °C).

3.2.2.6. Data analysis

All data are expressed as means ± SE; n indicates the number of animals studied. Statistical analysis of the data was performed using one-way or two-way ANOVA to detect differences among the means of the experimental groups. If significant differences were detected by ANOVA, pair-wise comparisons between means of the experimental groups were made using a Student's t test. A *P* value less than 0.05 is considered significantly different.

a) For vascular reactivity experiments, contractile responses to U-46619 or VP were normalized by dry weight of the aortic rings and were expressed as milligram tension per milligram ring weight (wt). The EC_{50}, defined as the effective concentration of VP or U-46619 causing 50% of the maximal contraction, was calculated from the individual concentration-response curve of each aortic ring. The EC_{50} is reported as the mean ± SE for the particular experimental group.

b) For TxA_2 and PGI_2 release experiments, basal release reflected the steady-state release of the prostanoids into the incubation medium (KHB) during the 45 minute incubation period, and was normalized by dry weight of aortic rings and expressed as pg/mg dry tissue weight/45 min. Agonist-stimulated release reflects the total agonist-stimulated release of the prostanoids into the incubation medium in the presence of a low

(1 x 10^{-8} M) or high (1 x 10^{-6} M) concentration of VP, as used in the contractile function experiments.

c) For the immunohistochemistry experiments, the amount of positive staining of each specific protein was visualized under light microscopy (10X) and was scored on a scale of 1 – 4 by a naïve individual blinded to the sex or treatment of the experimental animal. The weakest positive stain was scored as 1 and the strongest positive stain was scored as 4.

d) For RT-PCR experiments, the results were expressed as the light intensity ratio of the specific mRNA and the housekeeping gene GAPDH mRNA obtained from the same tissues.

3.3. Experimental design

3.3.1. Specific aim 1: To determine male-female differences in constrictor prostanoid function and vascular contraction in the rat aorta in vitro, and the role of estrogen in the regulation of these mechanisms

3.3.1.1. Rationale

Previous studies reveal that a marked sexual dimorphism exists in vascular reactivity of the rat aorta to VP. Maximal contractile response to VP is three-fold greater in the female than in the male aorta (70, 224, 225). Previous studies also established that VP-stimulated release of NO is much greater in the male rat aorta and is responsible for the attenuated responsiveness to VP. Interestingly, even in the presence of NOS inhibition, contractile responses of the rat aorta to VP are still significantly higher in

female, and the COX inhibitor indomethacin attenuates contractile responses to VP in the female but not male aorta (70, 225). These data suggest that constrictor products of COX may contribute to the enhanced vascular reactivity of the female rat aorta to VP. Therefore, in the present studies, the effects of COX inhibition on contractile responses of the female rat aorta to VP and the role of estrogen in regulating these responses to VP were examined. The role of estrogen in modulating constrictor prostanoid function was further examined by determining vascular reactivity to TxA_2 in the thoracic aortas of male and female rats.

3.3.1.2. Specific aim 1 experiments

Protocol 1: To determine the effects of cyclooxygenase inhibitors on contractile responses of female rat aorta to VP

The effects of prostanoid pathway inhibitors on vascular reactivity to VP were examined by obtaining cumulative concentration-responses to VP (1×10^{-11} - 1×10^{-6} M) in endothelium-intact (Endo+) aortas prepared in triplicate from InT-female rats in the presence of the following: *1*) The non-selective COX inhibitor indomethacin (Indo, 10 μM); *2*) The COX-2-selective inhibitor NS-398 (NS; 10 μM); or *3*) vehicle-control (0.13% DMSO in KHB). Aortic rings were pretreated with inhibitors or vehicle 20 min before the concentration-response to VP was obtained. To examine whether the effects of the COX-2-selective inhibitor were agent-specific, cumulative concentration-responses to VP in InT-female rats were also obtained in the presence of a second, chemically-dissimilar COX-2-selective inhibitor, niflumic acid (NA, 10 μM).

Protocol 2: To determine the effects of ovariectomy and estrogen replacement therapy on contractile responses of female rat aorta to VP

To determine the effects of estrogen on vascular reactivity and constrictor prostanoid function in the female rat aorta, cumulative concentration-responses to VP (10^{-11}-10^{-6} M) were obtained in Endo+ aortic rings prepared in triplicate from OvX-female, and OvX+Est-female rats. Rings from each aorta were pretreated with Indo (10 μM), NS (10 μM), or vehicle (control).

Protocol 3: To determine male-female differences in contractile responses to exogenous TxA_2 and the role of the endothelium

Male-female differences in contractile responses to TxA_2 and the role of the endothelium were determined by obtaining cumulative concentration-responses to the TxA_2 analog U-46619 (1×10^{-11} M - 3×10^{-6} M) in paired Endo+ and Endo- aortic rings obtained from male and InT-female rats.

Protocol 4: To determine the effects of estrogen on female vascular reactivity to U-46619

Cumulative concentration-responses to U-46619 (1×10^{-11} M - 3×10^{-6} M) were obtained in Endo+ aortic rings obtained from OvX-female and OvX+Est-female rats.

Protocol 5: To determine the effects of dietary phytoestrogens on the contractile responses of the rat aorta to VP and U-46619

The protein contents of regular rat chows are frequently enhanced by the addition of soybeans and alfalfa to the chow. These substances add significant amounts of phytoestrogen compounds to the diet. These compounds appear to exert estrogenic

actions at the estrogen receptor (2, 40, 59); therefore, the effects of phytoestrogens on vascular reactivity to VP and U-46619 were studied. 4-5 weeks old InT-female and OvX-female rats were fed a phytoestrogen-free diet in which casein replaced soy or alfalfa as the major source of dietary protein (7% corn oil diet, Harlan Tek-Lad) until they reach 14-15 weeks old. Aortic rings prepared from these phytoestrogen-free rats were used to obtain concentration-responses to VP and U-46619 in separate groups of female rats.

3.3.2. Specific aim 2: To determine male-female differences in TxA_2 and PGI_2 release by the rat aorta in vitro and the role of estrogen in the regulation of the TxA_2 and PGI_2 biosynthesis pathway

3.3.2.1. Rationale

TxA_2 and PGI_2 are both important vasoactive substances derived from the metabolism of AA in the vascular wall. The relative balance of their synthesis and release will result in a net increase or decrease in local vascular tone. Previous studies have established that sex differences exist in the contractile responses of the vasculature to VP and PE, and that male-female differences in agonist-induced TxA_2 function may be involved in these sex differences (70, 224, 225, 226, 227). Therefore, basal and agonist-induced release of TxA_2 and PGI_2 by male and female rat aortas were assessed by radioimmunoassay of their stable metabolites, TxB_2 and 6-keto-$PGF_{1\alpha}$ respectively. The effects of OvX and estrogen replacement therapy on the release of TxA_2 and PGI_2 by the female aorta were also determined.

3.3.2.2. Specific aim 2 experiments

Protocol 1: To determine male-female differences in basal and agonist-stimulated release of TxA_2 and PGI_2

Aortic rings, prepared in triplicate from InT-female or male rats, were incubated in 1.0 ml of fresh KHB either alone (basal) or with low-dose (10^{-8} M) VP or high-dose (10^{-6} M) VP, continuously gassed (95% O_2/5% CO_2) for 45 minutes at 37 °C. After incubation, the KHB was collected and stored at –70 °C until specific RIA of TxB_2 and 6-keto-$PGF_{1\alpha}$.

Protocol 2: To determine the effects of OvX and estrogen replacement therapy on the release of TxA_2 and PGI_2

Aortic rings from InT-female, OvX-female, or OvX+Est-female rats were prepared in triplicate to measure basal and low- or high-dose VP-stimulated release of TxA_2 and PGI_2 as described in Protocol 1.

Protocol 3: To determine the effects of COX and TxS inhibition and estrogen on the release of TxA_2 and PGI_2

Aortic rings prepared in quadruplicate from male, InT-female, OvX-female and OvX+Est-female rats were pre-incubated in either KHB alone or in the presence of Indo (10 μM), NS (10 μM), or dazoxiben (DAZ, TxS inhibitor, 50 μM). The rings were then incubated (45 min, 37 °C) with KHB containing 10^{-6} M VP either alone, or in the presence of 10 μM Indo, 10 μM NS or 50 μM DAZ, respectively. After incubation, KHB was collected, stored, and analyzed as described in protocol 1.

3.3.3. Specific aim 3: To determine the molecular mechanism(s) by which estrogen upregulates constrictor prostanoid function in the rat aorta

3.3.3.1. Rationale

Previous studies suggest that estrogen may enhance constrictor prostanoid pathway function, and thereby, potentiate contractile responses of the female rat aorta; however, the underlying mechanism(s) are still unclear. Estrogen may alter the expression of COX and/or TxS, thereby enhancing the production of TxA_2 and PGI_2. Estrogen may also affect the expression of TP by VSM cells to enhance the contractile responsiveness to TxA_2. Therefore, the expression of key prostanoid pathway enzyme messages for COX-1, COX-2 and TxS was determined by RT-PCR and the synthesis of the associated enzyme proteins were estimated by immunohistochemistry. Expression of TP message was also determined by RT-PCR. The effects of OvX and estrogen replacement therapy on the expression of these key prostanoid pathway enzyme mRNAs and their associated proteins were also examined.

3.3.3.2. Specific aim 3 experiments

Protocol 1: To determine the mRNA levels of COX-1, COX-2, and TxS by rat aorta and the effects of OvX and estrogen replacement therapy on their expression

Isolated aortic endothelium and VSM cells from male, InT-female, OvX-female, and OvX+Est-female rats were prepared to detect the mRNA levels of COX-1, COX-2, and TxS using the method of RT-PCR.

Protocol 2: To determine the protein expression of COX-1, COX-2 and TxS enzymes and the effects of OvX and estrogen replacement therapy on their expression

Aortic rings prepared from male, InT-female, OvX-female and OvX+Est-female rats were used for immunohistochemistry study to determine the protein expression of COX-1, COX-2, and TxS in aortic endothelium and VSM.

Protocol 3: To determine the expression of TxA_2 receptor (TP) mRNA by rat VSM cells and the effects of OvX and estrogen replacement therapy on TP expression

Isolated aortic VSM from male, InT-female, OvX-female and OvX+Est-female rats were prepared to detect the expression of TP mRNA levels by the method of RT-PCR.

CHAPTER IV

RESULTS

4.1. Specific aim 1: To determine male-female differences in constrictor prostanoid function and vascular contraction in the rat aorta in vitro, and the role of estrogen in the regulation of these mechanisms

4.1.1. Effects of COX inhibitors on contractile responses of female rat aorta to VP

In the InT-female rat aorta, both the non-selective COX inhibitor Indo and the COX-2-selective inhibitor NS-398 significantly attenuated contractile responses to VP at

Table 3. EC_{50} and corresponding maximal contractile responses to vasopressin in thoracic aortas of intact-, OvX-, and OvX+Est-female (F) Sprague-Dawley rats pretreated with indomethacin, NS-398 or vehicle-control. The OvX-female rats were fed with phytoestrogen-free diet.

Group	InT-F (n=5)		OvX-F (diet) (n=6)		OvX+Est-F (n=8)	
	EC_{50} (nM)	Maximal contractile force (mg/mg ring wt)	EC_{50} (nM)	Maximal contractile force (mg/mg ring wt)	EC_{50} (nM)	Maximal contractile force (mg/mg ring wt)
Cont	8.0 ± 1.2^{c}	$5{,}567\pm276^{g}$	9.2 ± 1.5^{c}	$2{,}485\pm394^{ef}$	4.9 ± 0.4^{a}	$5{,}059\pm194^{g}$
Indo	6.1 ± 0.4^{ac}	$3{,}176\pm179^{f}$	7.5 ± 0.7^{c}	$1{,}666\pm182^{e}$	5.6 ± 0.9^{ab}	$3{,}643\pm264^{f}$
NS	10.9 ± 1.6^{d}	$3{,}258\pm152^{f}$	9.0 ± 1.0^{c}	$1{,}833\pm171^{e}$	7.6 ± 0.4^{b}	$3{,}457\pm261^{f}$

Values are means ± SE; n, no. of animals. EC_{50}, concentration of agonist producing 50% of the maximal contractile response; Cont, vehicle-control; Indo, indomethacin (10 μm); NS, NS-398 (10 μm). $^{a\text{-}g}$ Within columns and rows for each group (intact *vs.* OvX *vs.* OvX+Est) or experimental treatment (control *vs.* Indo *vs.* NS), mean values for EC_{50} or maximal response without common superscript are significantly different ($P \leq 0.031$). Data are derived from Fig. 13, 16, 17.

middle and higher concentrations (Fig. 12, Table 3). The maximal contractile response was reduced by 43% (3,176 ± 179 mg/mg ring wt., $P < 0.001$) and 41% (3,258 ± 152 mg, $P < 0.001$) by Indo and NS respectively, compared to the vehicle-control group (5,567 ± 276 mg). Sensitivity (EC_{50}) of the female aorta to VP (8.0 ± 1.2 nM) was unchanged after pretreatment with either Indo (6.1 ± 0.4 nM, $P = 0.165$) or NS (10.9 ±

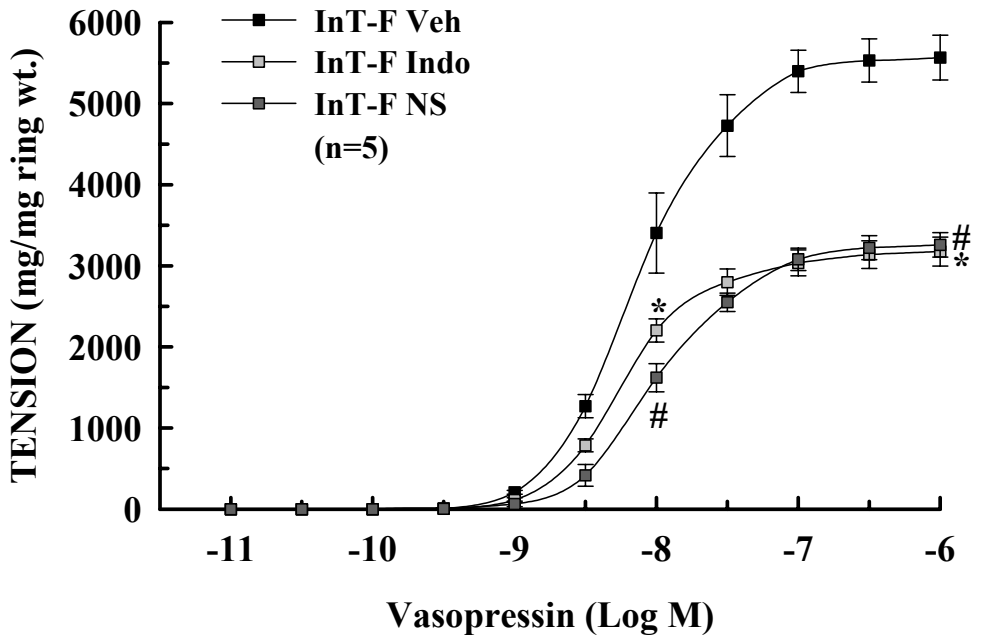

Fig. 12. Concentration-response curves for vasopressin in endothelium-intact aortic rings from intact (InT) female (F) Sprague-Dawley rats, in the presence of indomethacin (Indo, 10 μM), NS-398 (NS, 10 μM) or vehicle-control (Veh). Data points represent means ± SE (n, no. of animals). Statistically significant differences exist in Veh *vs*. Indo (*, $0.0001 \leq P \leq 0.048$) and Veh *vs*. NS ($^{\#}$, $0.0001 \leq P \leq 0.009$) at both maximal (1×10^{-6} M) and middle (1×10^{-8} M) concentrations of VP. A statistically significant difference exists in Indo *vs*. NS ($P = 0.032$) at middle but not maximal concentration of VP ($P = 0.736$).

1.6 nM, P = 0.180; Table 3). A chemically dissimilar COX-2-selective inhibitor, niflumic acid, produced similar attenuating effects (2,376 ± 265 mg, $P < 0.001$) as NS on the contractile responses to VP (Fig. 13).

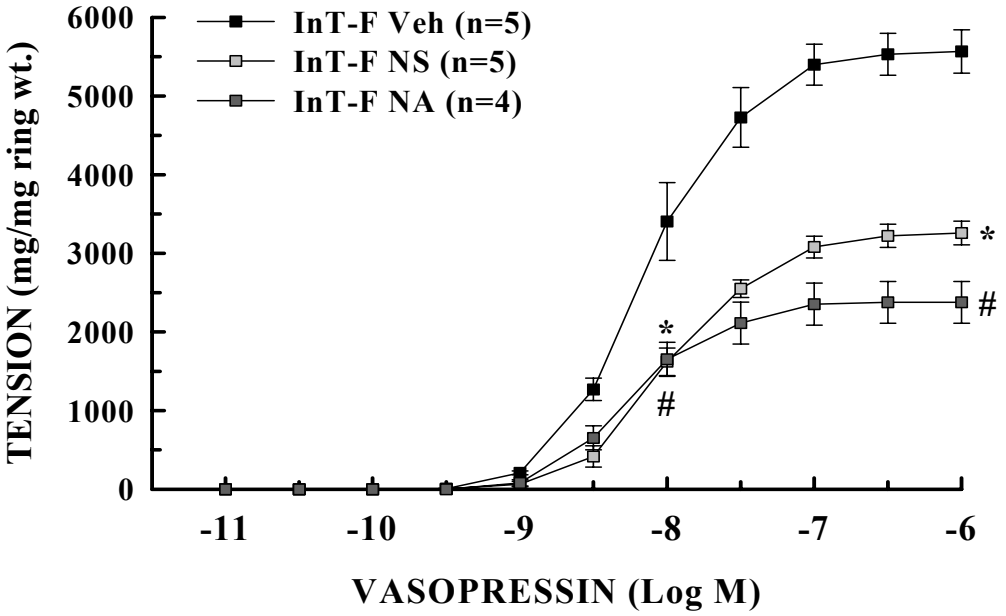

Fig. 13. Concentration-response curves for vasopressin in endothelium-intact aortic rings from intact (InT) female (F) Sprague-Dawley rats, in the presence of NS-398 (NS, 10 μM), niflumic acid (NA, 10 μM), or vehicle-control (Veh). Data points represent means ± SE (n, no. of animals). Statistically significant differences exist in Veh *vs*. NS (*, $0.048 \leq P \leq 0.0001$) and in Veh *vs*. NA ($^{\#}$, $0.021 \leq P \leq 0.004$) at both maximal (1×10^{-6} M) and middle (1×10^{-8} M) concentrations of VP.

4.1.2. Effects of OvX and estrogen replacement therapy on contractile responses of female rat aorta to VP

Plasma 17β-estradiol concentration of InT-female rats averaged 43.9 ± 13.0 pg/ml. OvX reduced plasma estradiol by 97% (1.3 ± 0.4 pg/ml, $P < 0.05$), whereas estrogen replacement therapy restored plasma 17β-estradiol (27.9 ± 5.6 pg/ml) to concentrations similar to those of InT-female rats (Table 4).

Table 4. Plasma estrogen concentrations of male and intact, OvX and OvX+Est-female rats.

Group	Male (n=6)	InT-F (n=5)	OvX-F (n=13)	OvX+Est-F (n=13)
Plasma estrogen level (pg/ml)	1.5 ± 0.5^{b}	43.9 ± 13.0^{a}	1.3 ± 0.4^{b}	27.9 ± 5.6^{a}

Values are means ± SE; *n,* no. of animals. InT-F, intact female; OvX-F, ovariectomized female; OvX+Est-F, OvX+17β-estradiol-replaced female. ^{a,b} mean values without common superscript are significantly different ($P < 0.05$).

In initial experiments with VP, OvX did not consistently alter contractile responses to VP, or the attenuating effects of Indo or NS. Similarly, in initial experiments with U-44619, OvX failed to attenuate the contractile responses of the female aorta to this agonist. These findings are consistent with previous studies using standard laboratory rat chow (70), and led to the consideration that dietary phytoestrogens, which are plant-derived, estrogen-like molecules, may be responsible for the inconsistency of the data. Phytoestrogens are reported to interact with the

estrogen receptor (135) and thus, may have masked the effects of OvX on vascular reactivity to VP and U-46619. As postulated, OvX-female rats fed with an alfalfa- and soy protein-free diet consistently exhibited attenuated contractile responses to VP throughout the concentration-response curve and the attenuating effects of Indo and NS were likewise abolished (Fig. 14, 15). In contrast, estrogen replacement therapy restored

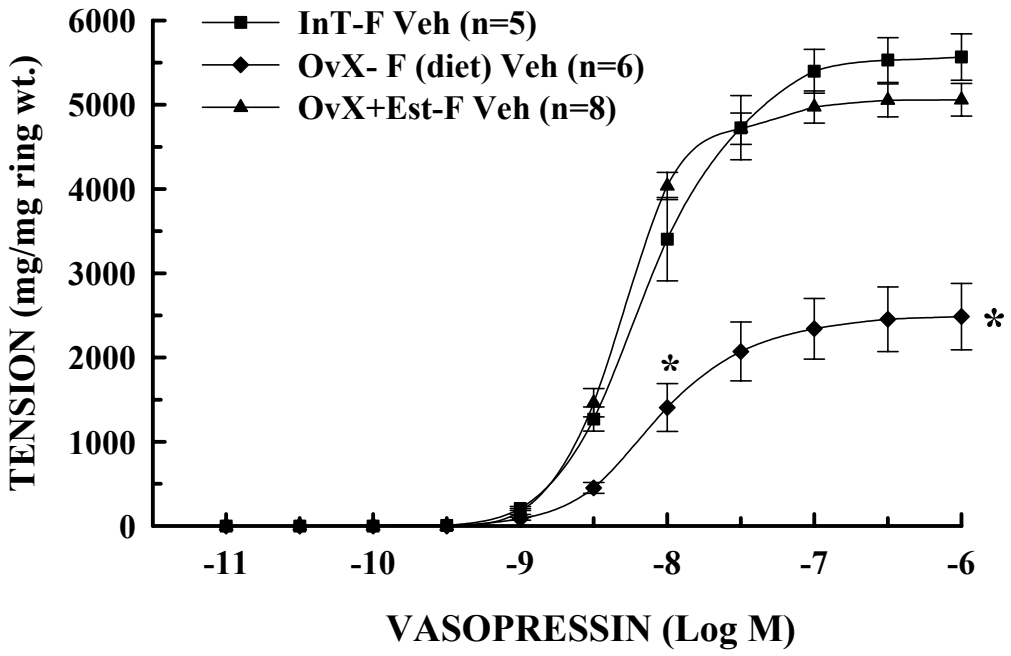

Fig. 14. Concentration-response curves for vasopressin in endothelium-intact aortic rings from intact- (InT), OvX-, and OvX+Est-female (F) Sprague-Dawley rats. The OvX-female rats were fed with phytoestrogen-free diet (diet). Data points represent means ± SE (n, no. of animals). Statistically significant differences exist in InT-F *vs*. OvX-F (*, $0.005 \leq P \leq 0.0002$) and OvX+Est-F *vs*. OvX-F (*, $P < 0.0001$) at maximal (1×10^{-6} M) and middle (1×10^{-8} M) concentrations of VP. No differences exist in InT-F *vs*. OvX+Est-F ($P > 0.1$).

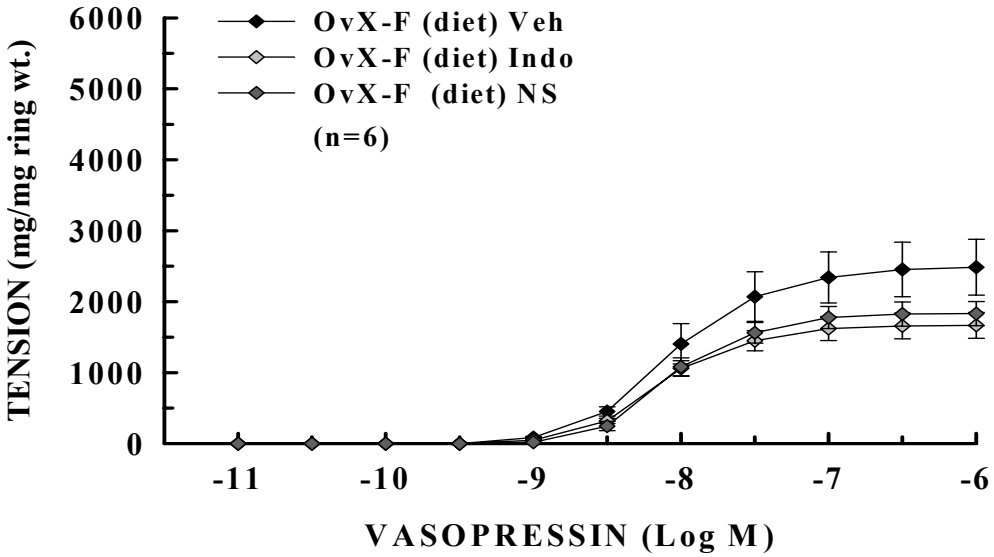

Fig. 15. Concentration-response curves for vasopressin in endothelium-intact aortic rings from OvX-female (F) Sprague-Dawley rats, in the presence of indomethacin (Indo, 10 μM), NS-398 (NS, 10 μM) or vehicle-control (Veh). The OvX-female rats were fed with phytoestrogen-free diet (diet). Data points represent means ± SE (n, no. of animals). No statistically significant differences exist in Veh *vs*. Indo ($P > 0.088$), Veh *vs*. NS ($P > 0.160$), or Indo *vs*. NS ($P > 0.521$) at both maximal (1×10^{-6} M) and middle (1×10^{-8} M) concentrations of VP.

the contractile responses to VP and the attenuating effects of Indo and NS (Fig. 14, 16). Maximal contractile responses to VP were reduced 55% by OvX (2,485 ± 394 mg, $P < 0.001$) and restored by estrogen replacement therapy (5,059 ± 194 mg, $P > 0.1$), compared with the InT-female aorta (Table 3). In the InT-female aorta, the differences in maximal contractile responses among control (5,567 ± 276 mg), Indo- (3,176 ± 179 mg), and NS-treated aortas (3,258 ± 152 mg) were highly significant ($P < 0.001$) (Table 3); in contrast, in the OvX-female aortas, maximal contractile responses of control (2,485 ±

394 mg), Indo- (1,666 ± 182 mg), and NS-treated aortas (1,833 ± 171 mg) did not differ significantly ($P > 0.05$) (Table 3). With estrogen replacement therapy, significant differences in maximal contractile responses among control (5,059 ± 194 mg), Indo- (3,643 ± 264 mg), and NS-treated aortas (3,457 ± 261 mg) were restored ($P < 0.001$). Sensitivity to VP (EC_{50}) was not altered by OvX and did not differ significantly among control, Indo-, or NS-treated aortas ($P > 0.1$; Table 3).

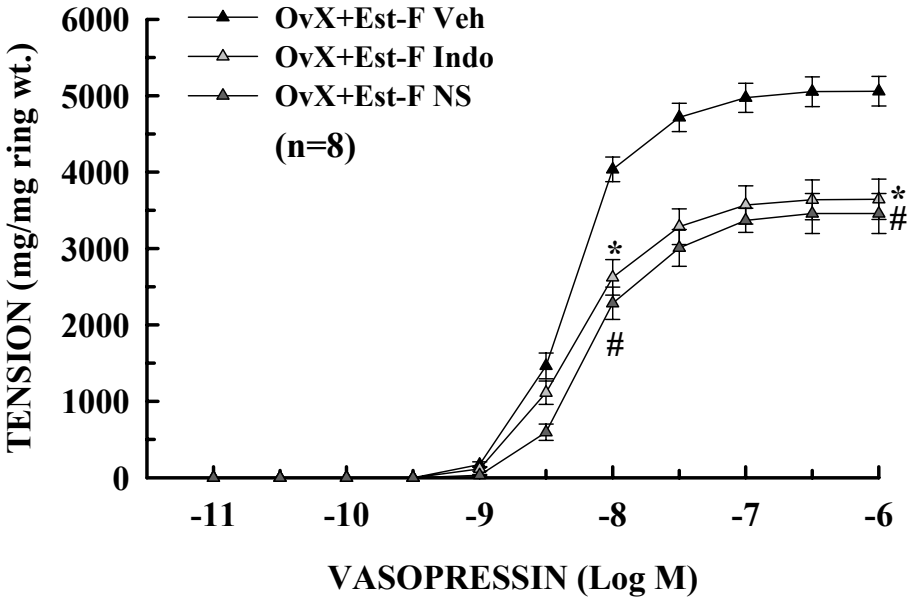

Fig. 16. Concentration-response curves for vasopressin in endothelium-intact aortic rings from OvX+Est-female (F) Sprague-Dawley rats, in the presence of indomethacin (Indo, 10 μM), NS-398 (NS, 10 μM) or vehicle-control (Veh). Data points represent means ± SE (n, no. of animals). Statistically significant differences exist in vehicle-control *vs*. Indo (*, $0.0002 \leq P \leq 0.0003$) and vehicle control *vs*. NS ($^{\#}$, $P < 0.0001$) at both maximal (1×10^{-6} M) and middle (1×10^{-8} M) concentrations of VP. No statistically significant differences exist in Indo *vs*. NS ($P > 0.299$).

4.1.3. Male-female differences in contractile responses to exogenous TxA_2 and the role of the endothelium

Contractile responses of Endo+ aortas to the stable TxA_2 analog U-46619 were significantly higher in female than in male in the upper half of the concentration-response curve (Fig. 17). The maximal contractile responses of the Endo+ aorta in female (5,040 ± 238 mg) were 40% higher than in male (3,679 ± 96 mg, $P < 0.0001$) (Fig. 17, Table 5). Deletion of the endothelium potentiated the contractile responses of

Table 5. EC_{50} and corresponding maximal contractile responses to the thromboxane analog U-46619 in paired endothelium-intact and endothelium-denuded thoracic aortic rings of male, and intact-, OvX-, and OvX+Est-female (F) Sprague-Dawley rats.

	Male (n=9)		InT-F (n=8)		OvX-F (diet) (n=7)		OvX+Est-F (n=5)	
Group	EC_{50} (nM)	Maximal contractile force (mg/mg ring wt)	EC_{50} (nM)	Maximal contractile force (mg/mg ring wt)	EC_{50} (nM)	Maximal contractile force (mg/mg ring wt)	EC_{50} (nM)	Maximal contractile force (mg/mg ring wt)
Endo+	27.3 ± 3.2^{cd}	3,679 ± 95.8^{e}	34.7 ± 2.2^{d}	5,040 ± 238^{h}	30.3 ± 2.8^{d}	3,923 ± 84.1^{e}	38.5 ± 9.3^{d}	5,024 ± 155^{h}
Endo-	13.0 ± 1.7^{a}	4,099 ± 174.4^{ef}	20.4 ± 2.6^{bc}	5,599 ± 227^{h}	14.8 ± 3.0^{ab}	4,441 ± 99.6^{fg}	35.2 ± 2.4^{d}	4,976 ± 353^{gh}

Values are means ± SE (n, no. of animals). EC_{50}, concentration of agonist producing 50% of the maximal contractile response; Endo+, endothelium-intact; Endo-, endothelium-denuded. $^{a-g}$ within columns and rows for each group (Male *vs*. InT-F *vs*. OvX-F *vs*. OvX+Est-F) or experimental treatment (Endo+ *vs*. Endo-), mean values for EC_{50} or maximal response without common superscript are significantly different ($P \leq 0.029$). Data are derived from Fig. 17, 18.

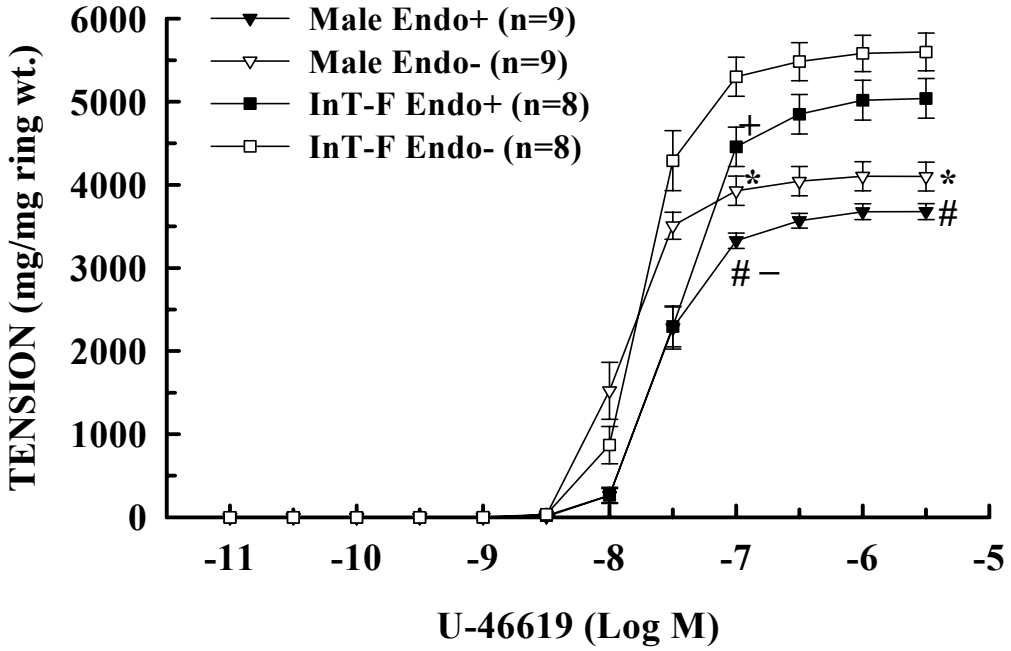

Fig. 17. Concentration-response curves for U-46619 in endothelium-intact (Endo+) and endothelium-denuded (Endo-) aortic rings from intact (InT) female (F) and male (M) Sprague-Dawley rats. Data points represent means ± SE (n, no. of animals). Statistically significant differences exist in InT-F Endo+ *vs*. M Endo+ (*, $P < 0.001$) and InT-F Endo- *vs*. M Endo- ($^{\#}$, $P < 0.001$) at both maximal (3 x 10^{-6} M) and middle (1 x 10^{-7} M) concentrations of U-46619. No statistically significant differences exist in Endo+ F *vs*. Endo- F ($P = 0.111$) and Endo+ M *vs*. Endo- M ($P = 0.051$) at maximal concentrations of U-46619. Statistically significant differences exist in Endo+ F *vs*. Endo- F ($^{+}$, $P = 0.012$) and Endo+ M *vs*. Endo- M ($^{-}$, $P = 0.008$) at middle concentrations of U-46619.

both female and male rat aortas to a similar extent; thus, contractile responses of Endo- female (5,599 ± 227 mg) were 37% greater than those of Endo- male (4,099 ± 174 mg, $P < 0.0001$) (Fig. 17, Table 5). Sensitivity (EC_{50}) to U-46619 in the Endo+ aorta did not differ significantly between female (34.7 ± 2.2 nM) and male (27.3 ± 3.2 nM, $P > 0.05$). Deletion of the endothelium significantly increased the sensitivity of both female (20.4 ±

2.6 nM, $P = 0.0009$) and male (13.0 ± 1.7 nM, $P = 0.0012$) aortas, and the sensitivity to U-46619 in Endo- aorta was higher in male than in InT-female ($P = 0.028$) (Table 5).

4.1.4. Effects of estrogen on female vascular reactivity to U-46619

OvX noticeably attenuated the contractile responses of the female Endo+ aorta to U-46619. The maximal response to U-46619 was attenuated by 22% (3,923 ± 84 mg, $P < 0.01$) (Fig. 18, Table 5), compared to the InT-female aorta (5,040 ± 238 mg). Estrogen replacement therapy restored the contractile responses to U-46619 (4,976 ± 353 mg) in Endo+ aorta to levels similar to those of InT-female rat aortas (Fig. 18, Table 5). OvX had no effects on the sensitivity (EC_{50}) of the Endo+ aorta to U-46619 ($P > 0.1$) (Table 5).

4.1.5. Effects of dietary phytoestrogens on the contractile responses of the rat aorta to VP and U-46619

In OvX-female rats that were fed with standard rat chow diet, contractile responses to U-46619 fell between the curves for InT-female and male at middle and higher concentrations (Fig. 19). The maximal contractile response of the OvX-female aorta (4,472 ± 163 mg) was reduced by 11%, but was not significantly different from that of the InT-female aorta (5,040 ± 238 mg, $P = 0.063$) but was significantly higher than that of the male aorta (3,679 ± 95.8 mg, $P < 0.001$). In order to examine the role of male sex steroids in regulating the contractile responses to U-46619, male rats were

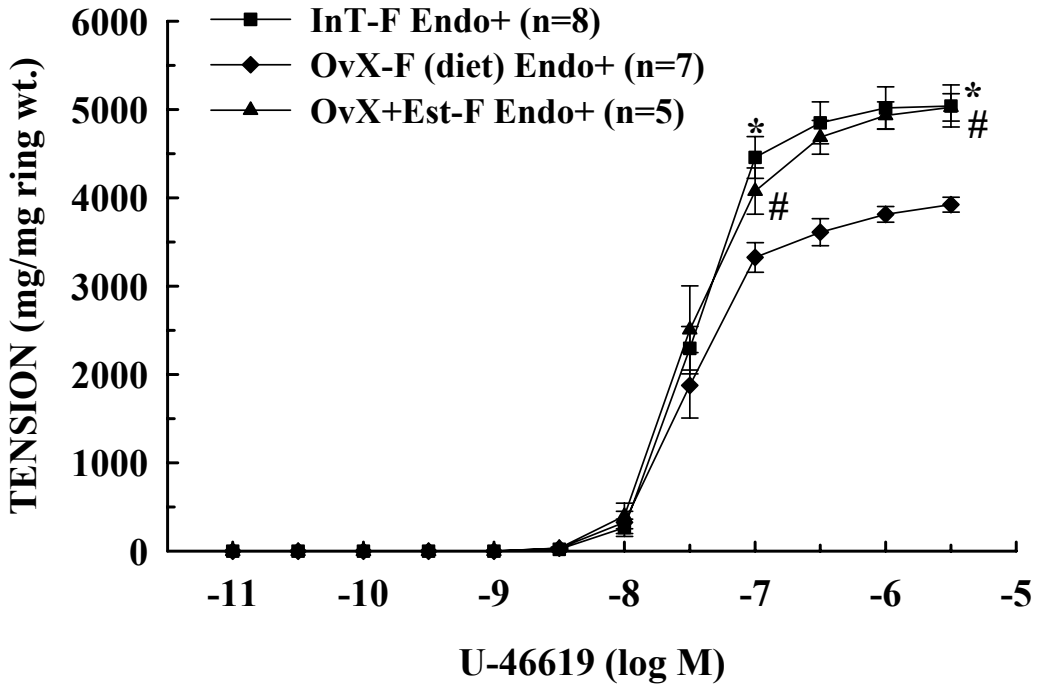

Fig. 18. Concentration-response curves for U-46619 in endothelium-intact (Endo+) aortic rings from intact- (InT), OvX-, and OvX+Est-female (F) Sprague-Dawley rats. The OvX-female rats were fed with phytoestrogen-free diet (diet). Data points represent means ± SE (n, no. of animals). Statistically significant differences exist in InT-F *vs*. OvX-F (*, $0.001 \leq P \leq 0.002$) and OvX+Est-F *vs*. OvX-F ($^{\#}$, $0.031 \leq P \leq 0.0001$) at both maximal (3×10^{-6} M) and middle (1×10^{-7} M) concentrations of U-46619. No statistically significant differences exist in InT-F *vs*. OvX+Est-F ($0.307 \leq P \leq 0.963$) at both maximal (3×10^{-6} M) and middle (1×10^{-7} M) concentrations of U-46619.

castrated at 4 weeks age and a concentration-response to U-46619 was obtained at 14-18 weeks age (as in intact male rats). Surprisingly, contractile responses of both Endo+ and Endo- male aortas to U-46619 were unaffected by castration (Fig. 20). In contrast, the phytoestrogen-free diet further attenuated contractile responses of the OvX-female aorta to U-46619 at both middle and maximal concentrations by 16% and 22%, respectively,

so that OvX-female response did not differ from those of male aorta, but were significantly lower than responses of InT-female aorta ($P < 0.01$, Fig. 19). The maximal contractile response to U-46619 in the OvX-female aorta on the standard diet and on the special diet did not differ significantly (4,472 ± 163 mg *vs*. 4,182 ± 95.3 mg respectively, $P > 0.05$). InT-female rats fed with the phytoestrogen-free diet until 14-18

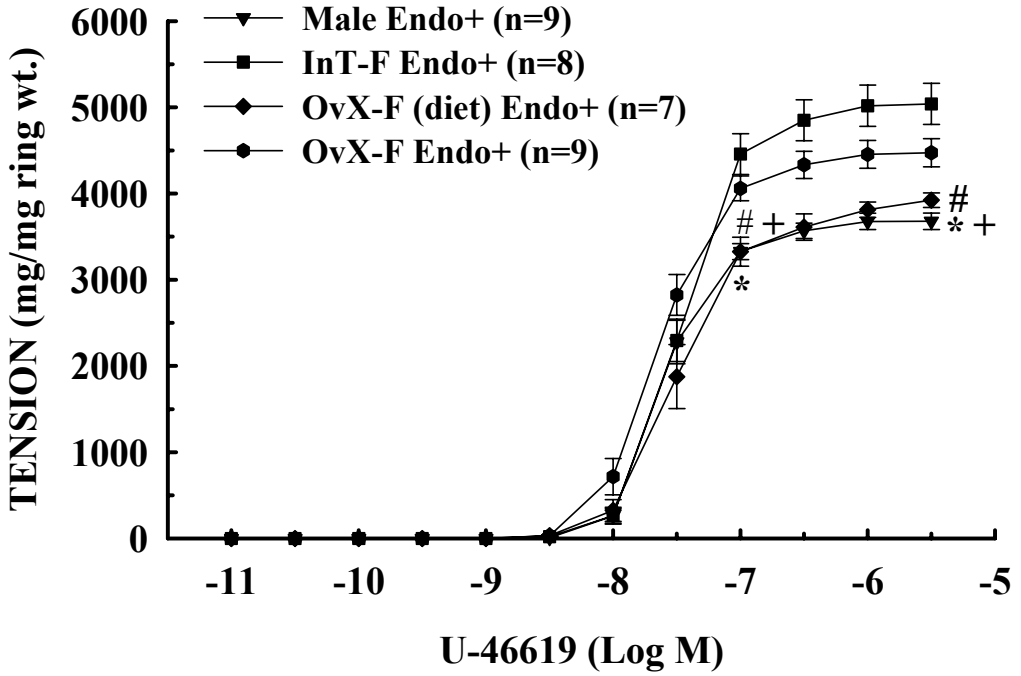

Fig. 19. Concentration-response curves for U-46619 in endothelium-intact (Endo+) aortic rings from male (M), intact- (InT), and OvX-female (F) Sprague-Dawley rats fed with standard rat chow or phytoestrogen-free diet (diet). Data points represent means ± SE (n, no. of animals). Statistically significant differences exist in InT-F *vs*. M (*, $P < 0.001$), InT-F *vs*. OvX-F fed with special diet ($^{\#}$, $P < 0.01$), and OvX-F fed with standard rat chow *vs*. M ($^{+}$, $P < 0.001$) at maximal (3×10^{-6} M) concentrations of U-46619. No statistically significant differences exist in InT-F *vs*. OvX-F fed with standard rat chow ($P = 0.063$) at maximal (3×10^{-6} M) concentrations of U-46619.

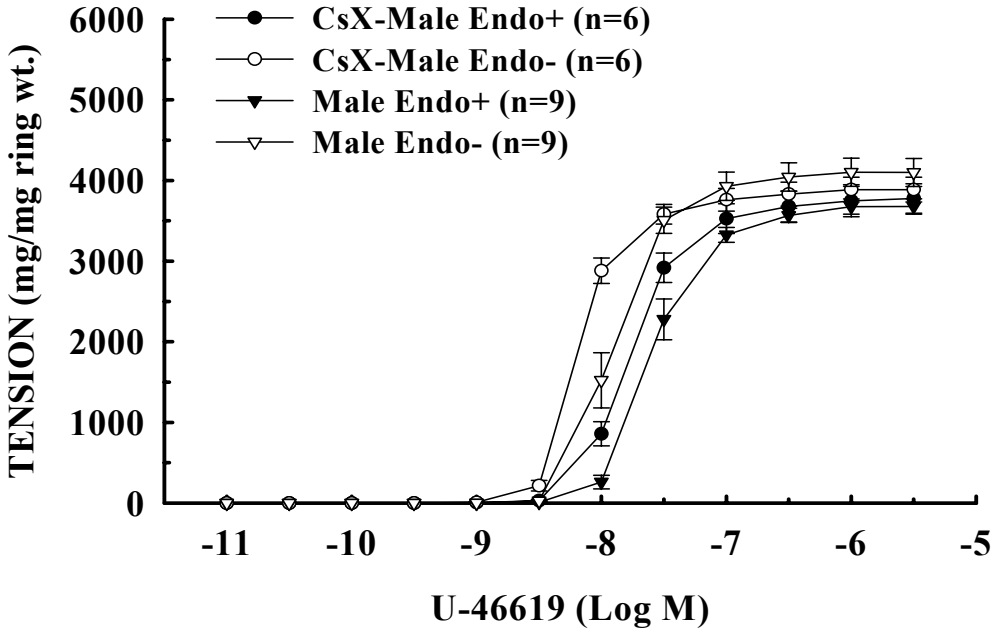

Fig. 20. Concentration-response curves for U-46619 in endothelium-intact (Endo+) and endothelium-denuded (Endo-) aortic rings from intact (InT) and castrated (CsX) male (M) Sprague-Dawley rats. Data points represent means ± SE (n, no. of animals). No statistically significant differences exist in Endo+ M *vs*. Endo+ CsX-M (P =0.612)), Endo- M *vs*. Endo- CsX-M (P = 0.408) or Endo+ CsX-M *vs*. Endo- CsX-M (P = 0.653), at maximal concentrations of U-46619.

weeks age exhibited slightly lower contractile responses to U-46619 compared to the InT-female rats fed with standard diet, but the differences were not significant (4,629 ± 158 mg *vs.* 5,040 ± 238 mg, respectively, $P > 0.1$).

The effects of the phytoestrogen-free diet on the contractile responses of female rat aorta to VP were discussed in section 4.1.2.

4.2. Specific aim 2: To determine male-female differences in TxA_2 and PGI_2 release by the rat aorta in vitro and the role of estrogen in the regulation of the TxA_2 and PGI_2 biosynthesis pathway

4.2.1. Male-female differences in basal and agonist-stimulated release of TxA_2 and PGI_2

Basal release of TxB_2 did not differ significantly between InT-female (20.2 ± 4.2 pg/mg ring wt./45 min) and male (18.8 ± 1.9 pg, $P = 0.388$) rat aortas (Fig. 21, Table 6). VP stimulated TxB_2 release in a concentration-dependent manner, and both low (1×10^{-8} M and high (1×10^{-6} M) concentrations of VP stimulated significantly more release of TxB_2 by female than by male rat aortas. In InT-female, low and high concentration of VP increased TxB_2 release by 121% (44.6 ± 7.0 pg, $P = 0.006$) and 334% (87.7 ± 12.7 pg, $P < 0.001$), respectively, compared to basal release (20.2 ± 4.2 pg) (Fig. 21, Table 6). In contrast, in the male aorta, the low concentration of VP increased TxB_2 release by only 37% (25.7 ± 4.2 pg), and the high concentration of VP increased TxB_2 release by only 134% (43.9 ± 8.3 pg). The low-dose VP-stimulated release of TxB_2 in the male aorta did not differ significantly from the basal group (18.8 ± 1.9 pg, $P = 0.08$); whereas the high VP-stimulated release of TxB_2 did differ from the basal group ($P = 0.014$).

Table 6. Basal and low (10^{-8} M) or high (10^{-6} M) concentration VP-stimulated release of TxB_2 and 6-keto-$PGF_{1\alpha}$ from male, intact- (InT), OvX-, and OvX+Est-female (F) rat aortas.

Group	TxB_2 (pg/mg ring wt./45 min)			6-keto-$PGF_{1\alpha}$ (pg/mg ring wt./45 min)		
	Basal	Low-conc. VP (10^{-8} M)	High-conc. VP (10^{-6} M)	Basal	Low-conc. VP (10^{-8} M)	High-conc. VP (10^{-6} M)
InT-F (n=7)	20.2±4.2^{ab}	44.6±7.0^{c}	87.7±12.7^{d}	1,488±123^{a}	3,860±800^{bc}	12,646± 2,683^{d}
Male (n=6)	18.8±1.9^{ab}	25.7±4.2^{b}	43.9±8.3^{c}	1,723±153^{a}	2,811± 184^{b}	5,155± 1,046^{c}
OvX-F (n=6)	17.4±1.7^{a}	23.7±2.3^{b}	38.2±4.2^{c}	1,316±128^{a}	2,107± 38^{ab}	4,228± 520^{c}
OvX+Est-F (n=6)	28±2.4^{b}	56.9±9.3^{c}	91±9.5^{d}	1,746±415^{a}	3,807±790^{bc}	16,071± 2,247^{d}

Values are means ± SE (n, no. of animals). $^{a\text{-}d}$ for each animal group (InT-F *vs*. Male *vs*. OvX-F *vs*. OvX+Est-F) and for each treatment group (Basal *vs*. Low-dose *vs*. High-dose), mean values for TxB_2 or 6-keto-$PGF_{1\alpha}$ without common superscript are significantly different ($0.05 > P > 0.0001$). conc., concentration. Data are derived from Fig. 21, 22.

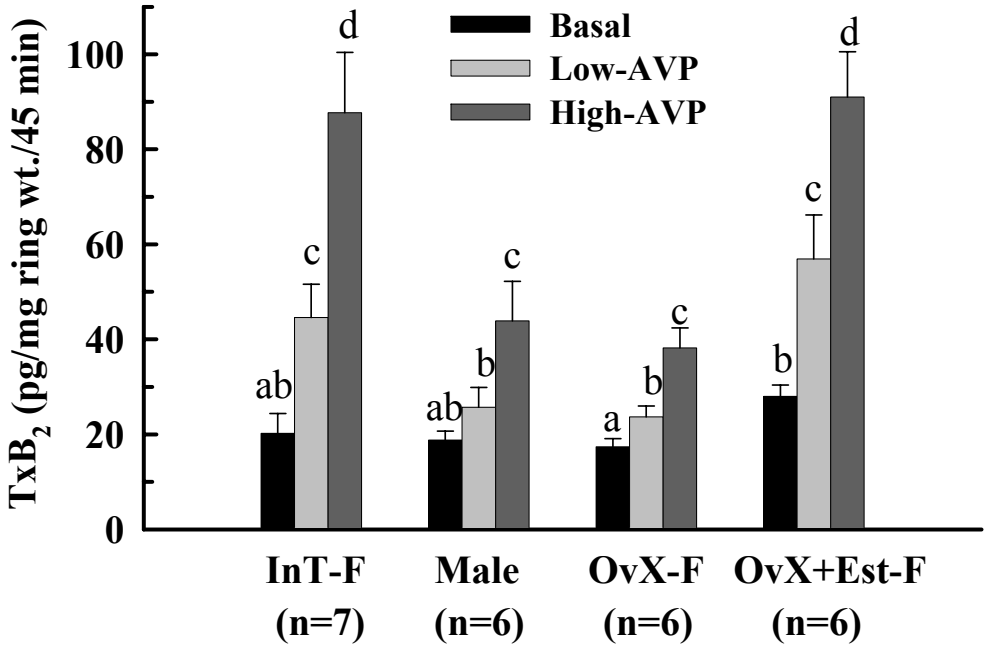

Fig. 21. Basal and VP-stimulated (Low-dose, 10^{-8} M, or high-dose, 10^{-6} M) release of TxB_2 by aortic rings prepared from male, intact- (InT), OvX- and OvX+Est-female (F) rats. Data points represent means ± SE (n, no. of animals). [a-d] For each group (male *vs*. InT-F *vs*. OvX-F *vs*. OvX+Est-F) and treatment (basal *vs*. Low *vs*. High), mean values for TxB_2 release without common superscript are significantly different ($0.05 > P > 0.0001$).

Basal release of 6-keto-$PGF_{1\alpha}$ did not differ significantly between InT-female (1,488 ± 123 pg/mg ring wt./45 min) and male (1,723 ± 153 pg, $P = 0.388$) rat aorta (Fig. 22, Table 6). VP also stimulated the release of 6-keto-$PGF_{1\alpha}$ in a concentration-dependent manner. Compared to basal, both low and high concentrations of VP stimulated significantly more release of 6-keto-$PGF_{1\alpha}$ in both InT-female and male rat

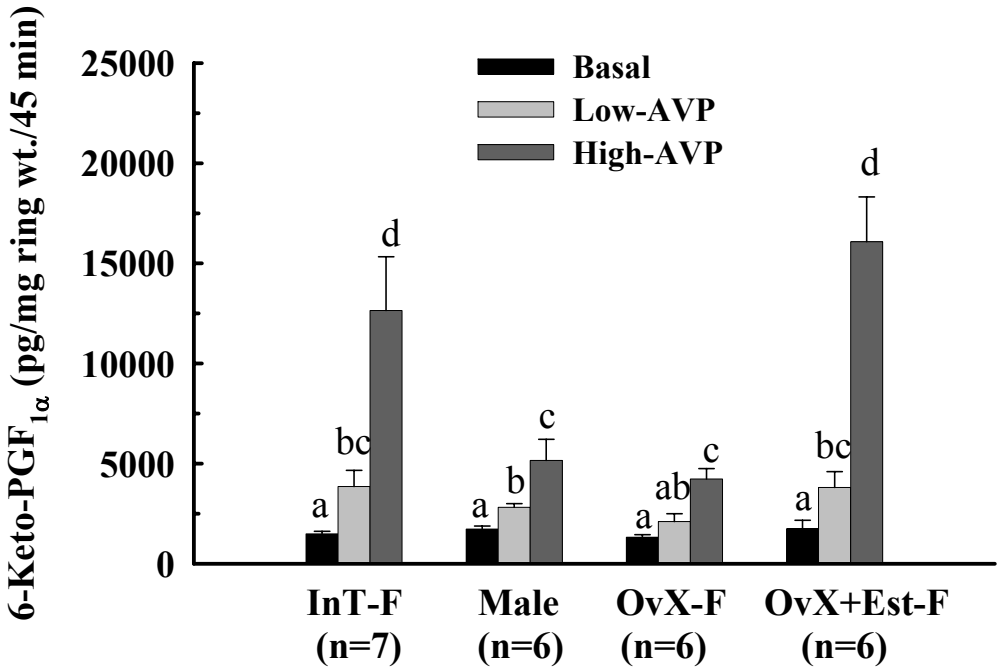

Fig. 22. Basal and VP-stimulated (Low-dose, 10^{-8} M, or high-dose, 10^{-6} M) release of 6-keto-$PGF_{1\alpha}$ by aortic rings prepared from male, intact- (InT), OvX- and OvX+Est-female (F) rats. Data points represent means ± SE (n, no. of animals). [a-d] For each group (male *vs*. InT-F *vs*. OvX-F *vs*. OvX+Est-F) and treatment (basal *vs*. Low *vs*. High), mean values for 6-keto-$PGF_{1\alpha}$ release without common superscript are significantly different ($0.05 > P > 0.0001$).

aortas, although responses of female aorta were substantially greater. In all four experimental groups, VP stimulated the release of substantially more 6-keto-$PGF_{1\alpha}$ than TxB_2 (Table 6). In InT-female, low and high concentrations of VP increased the release of 6-keto-$PGF_{1\alpha}$ by 159% (3,860 ± 800 pg, $P = 0.013$) and 750% (12,646 ± 2683 pg, $P < 0.001$), respectively, compared to the basal group (1,488 ± 123 pg) (Fig. 22, Table 6). In male, low and high concentrations of VP increased the release of 6-keto-$PGF_{1\alpha}$ by 63%

(2,811 ± 184 pg, P = 0.002) and 199% (5,155 ± 1,046 pg, P = 0.012), respectively, compared to the basal group.

4.2.2. Effects of OvX and estrogen replacement therapy on the release of TxA_2 and PGI_2

OvX and estrogen replacement therapy had no effects on basal release of either TxB_2 or 6-keto-$PGF_{1\alpha}$ (Fig. 21, 22, Table 6). In contrast, OvX significantly decreased both low- (23.7 ± 2.3 pg, P = 0.011) and high- (38.2 ± 4.2 pg, P = 0.003) concentration VP-stimulated release of TxB_2 to levels similar to those of male rats, while estrogen replacement therapy restored both low- and high-concentrations VP-stimulated release of TxB_2 to levels not different from those of InT-female (Fig. 21, Table 6). Low-concentration VP-stimulated release of 6-keto-$PGF_{1\alpha}$, while reduced by OvX and increased by estrogen replacement therapy, did not differ significantly between InT-female (3,860 ± 800 pg) and OvX-female (2,107 ± 386 pg, P = 0.089) or between OvX-female and OvX+Est-female (3,807 ± 790 pg, P = 0.082) rat aortas (Fig. 22, Table 6). High concentration VP-stimulated release of 6-keto-$PGF_{1\alpha}$ was reduced significantly (67%) by OvX (4,228 ± 520 pg, P = 0.016) to levels similar to those of male rats (5,155 ± 1046 pg) and was restored by estrogen replacement therapy (16,071 ± 2,247 pg) to levels similar to those of the InT-female aorta (Fig. 22, Table 6).

4.2.3. Effects of COX and TxS inhibition and estrogen on the release of TxA_2 and PGI_2

The non-selective COX inhibitor Indo (10 μM) and the COX-2-selective inhibitor NS-398 (10 μM) both significantly inhibited the release of TxB_2 and 6-keto-$PGF_{1\alpha}$ from male, InT-female and OvX-female rat aortas (Fig. 23, 24, Table 7). Indo inhibited TxB_2 release to a similar extent in InT-female (91% ± 3%), male (95% ± 2%) and OvX-female (86% ± 4%) aortas ($P > 0.08$). NS inhibited TxB_2 release to a similar

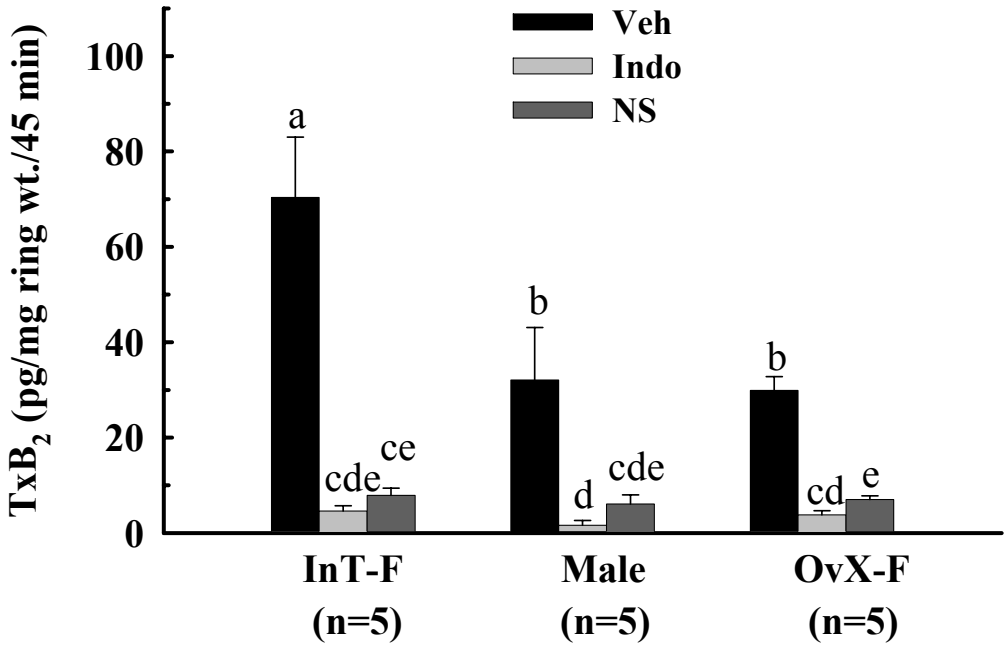

Fig. 23. High-dose (10^{-6} M) VP-stimulated release of TxB_2 by aortic rings prepared from InT-F, male and OvX-F rats, in the presence of indomethacin (Indo, 10 μM), NS-398 (NS, 10 μM) or vehicle-control (Veh). Data bars represent means ± SE (n, no. of animals). [a-e] For each group (InT-F *vs*. male *vs.* OvX-F) and treatment (Veh *vs*. Indo *vs*. NS), mean values for TxB_2 release without common superscript are significantly different ($0.0001 \leq P \leq 0.03$).

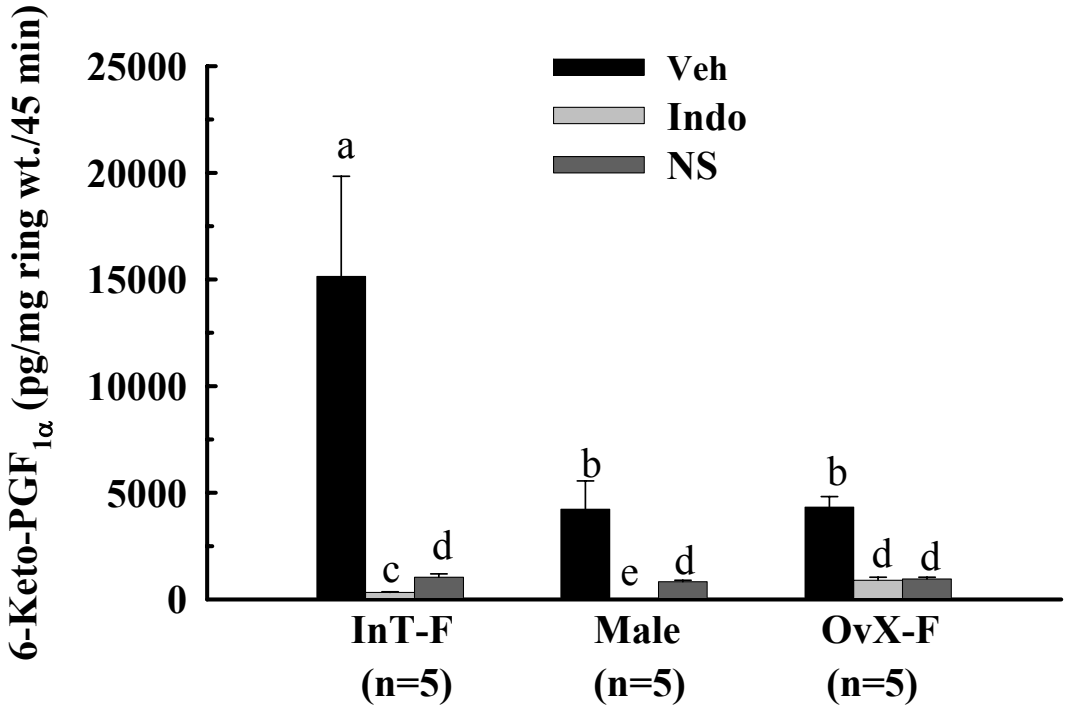

Fig. 24. High-dose (10^{-6} M) VP-stimulated release of 6-keto-$PGF_{1\alpha}$ by aortic rings prepared from InT-F, male and OvX-F rats, in the presence of indomethacin (Indo, 10 μM), NS-398 (NS, 10 μM) or vehicle-control (Veh). Data bars represent means ± SE (n, no. of animals). [a-d] For each group (InT-F *vs*. male *vs.* OvX-F) and treatment (Veh *vs*. Indo *vs*. NS), mean values for 6-keto-$PGF_{1\alpha}$ release without common superscript are significantly different ($0.0001 \leq P \leq 0.004$).

extent in InT-female (90% ± 3%) and male (86% ± 4%) (P = 0.44), but inhibited significantly less TxB_2 release in OvX-female (76% ± 3%) compared to InT-female (P = 0.009) or male aortas (P = 0.033). Indo inhibited significantly more 6-keto-$PGF_{1\alpha}$ release in InT-female (97% ± 1%) and male (99% ± 0%) than in OvX-female (78% ± 5%) ($P < 0.002$). NS inhibited more 6-keto- $PGF_{1\alpha}$ release in InT-female (84%) than in male (69%, P = 0.002) or OvX-female (76%, P = 0.086).

Table 7. High (10^{-6} M) concentration of VP-stimulated release of TxB_2 and 6-keto-$PGF_{1\alpha}$ from male, intact-, and OvX-female (F) rat aortas in the presence of vehicle-control (Veh), indomethacin (Indo, 10 μM), or NS-398 (NS, 10 μM).

Group	TxB_2 (pg/mg ring wt./45 min)			6-keto-$PGF_{1\alpha}$ (pg/mg ring wt./45 min)		
	Veh	Indo (10 μM)	NS (10 μM)	Veh	Indo (10 μM)	NS (10 μM)
InT-F (n=5)	70.4±12.6[d]	4.6±1.1[a]	7.9±1.5[b]	15,134±4,699[e]	323±34.2[b]	1,040±151[c]
Male (n=5)	32.1±11[c]	1.6±1.0[a]	6.1±1.9[b]	4,223±1,329[d]	11.0±4.1[a]	825±72.7[c]
OvX-F (n=5)	29.9±2.9[c]	3.8±0.9[a]	7.0±0.8[b]	4,328±484[d]	891.4±139[c]	958±79.1[c]

Values are means ± SE (n, no. of animals). [a-d] for each animal group (InT-F *vs*. Male *vs*. OvX-F) and for each treatment group (Veh *vs*. Indo *vs*. NS), mean values for TxB_2 and 6-keto-$PGF_{1\alpha}$ without common superscript are significantly different ($0.0001 \leq P \leq 0.04$). Data are derived from Fig. 23, 24.

The TxS inhibitor DAZ (50 μM) inhibited high-concentration VP-stimulated TxB_2 release from male, InT-female, OvX-female and OvX+Est-female rat aortas significantly ($P \leq 0.008$) (Fig. 25, Table 8). In contrast, DAZ increased the release of 6-keto-$PGF_{1\alpha}$ in all four experimental groups significantly ($P \leq 0.015$) (Fig. 26, Table 8). The inhibitory effect of DAZ on TxB_2 release was slightly higher in InT-female (79% ± 3%) and OvX+Est-female (77% ± 3%) than in male (74% ± 2%) and OvX-female (70% ± 9%), but the differences were not statistically significant ($P > 0.11$). Interestingly, DAZ did not decrease, but rather increased 6-keto- $PGF_{1\alpha}$ release in all four experimental groups. DAZ only increased 6-keto-$PGF_{1\alpha}$ release by 66% ± 11% in InT-female and 42% ± 17% in OvX+Est-female, respectively ($P = 0.12$), while it increased

6-keto- $PGF_{1\alpha}$ release by 298% ± 52% in male and by 178% ± 57% in OvX-female, which were markedly higher than InT-female ($P \leq 0.015$).

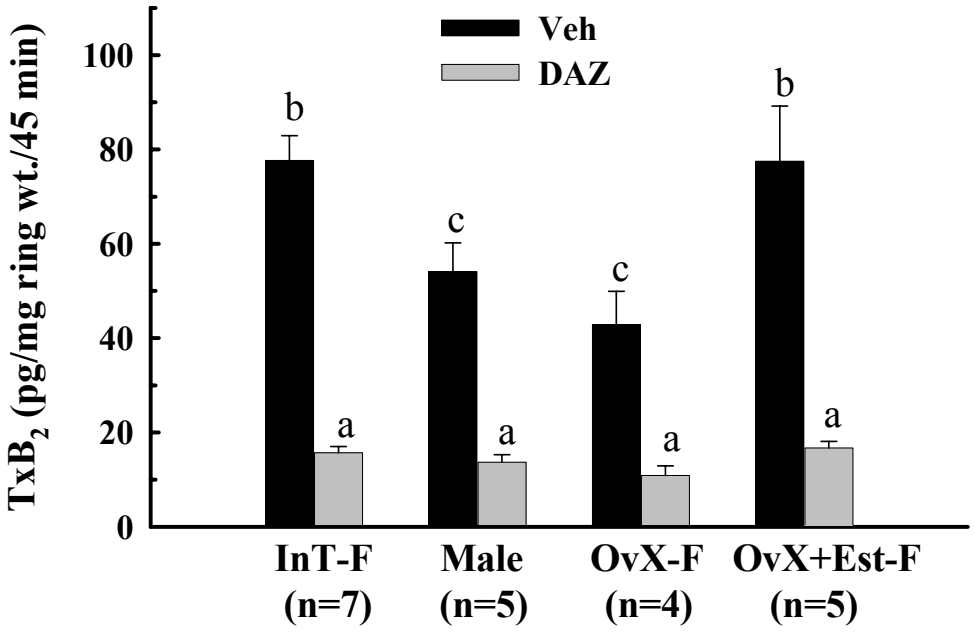

Fig. 25. High-dose (10^{-6} M) VP-stimulated release of TxB_2 by aortic rings prepared from male, InT-F, OvX-F, and OvX+Est-F rats, in the presence of dazoxiben (DAZ, 50 μM) or vehicle-control (Veh). Data bars represent means ± SE (n, no. of animals). [a-c] For each group (InT-F *vs*. male *vs.* OvX-F *vs*. OvX+Est-F) and treatment (Veh *vs*. DAZ), mean values for TxB_2 release without common superscript are significantly different ($0.0001 \leq P \leq 0.03$).

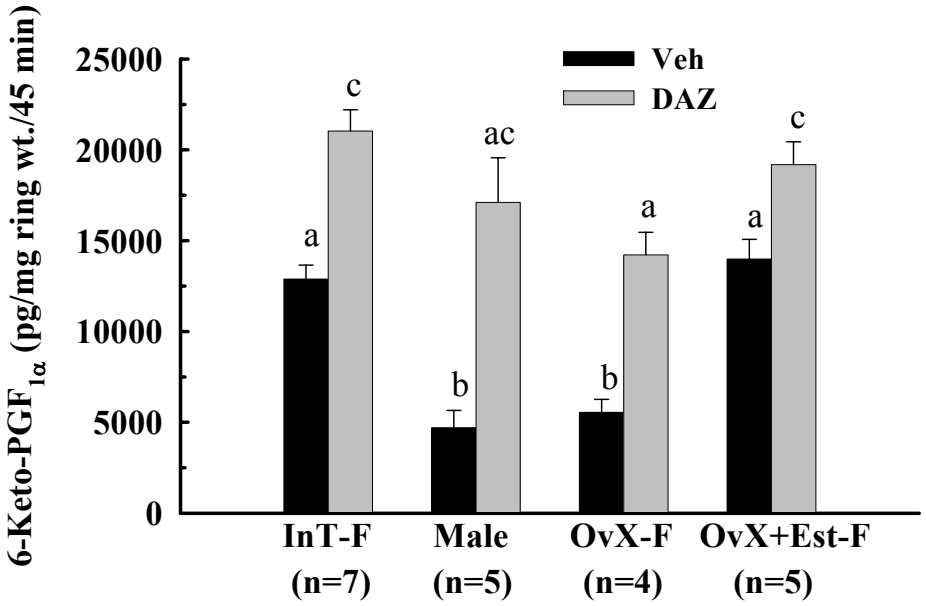

Fig. 26. High-dose (10^{-6} M) VP-stimulated release of 6-keto-$PGF_{1\alpha}$ by aortic rings prepared from male, InT-F, OvX-F, and OvX+Est-F rats, in the presence of dazoxiben (DAZ, 50μM) or vehicle-control (Veh). Data bars represent means ± SE (n, no. of animals). [a-c] For each group (InT-F *vs*. male *vs.* OvX-F *vs*. OvX+Est-F) and treatment (Veh *vs*. DAZ), mean values for 6-keto-$PGF_{1\alpha}$ release without common superscript are significantly different ($0.0001 \leq P \leq 0.033$).

Table 8. High (10^{-6} M) concentration of VP-stimulated release of TxB_2 and 6-keto-$PGF_{1\alpha}$ from male and InT-, OvX-, and OvX+Est-female (F) rat aortas in the presence of vehicle-control (Veh), or dazoxiben (DAZ, 50 μM).

Group	TxB_2 (pg/mg ring wt./45 min)		6-keto-$PGF_{1\alpha}$ (pg/mg ring wt./45 min)	
	Veh	DAZ (50 μM)	Veh	DAZ (50 μM)
InT-F (n=7)	77.7±5.2[c]	15.7±1.3[a]	12,888±765[e]	21,039±1,168[f]
Male (n=5)	54.1±6.1[b]	13.7±1.6[a]	4,703±956[d]	17,111±2,457[ef]
OvX-F (n=4)	42.9±7[b]	10.9±2[a]	5,540±720[d]	14,219±1,237[e]
OvX+Est-F (n=5)	77.5±11.7[c]	16.7±1.4[a]	13,983±1,094[e]	19,180±1,265[f]

Values are means ± SE (n, no. of animals). [a-c] for each animal group (InT-F *vs*. Male *vs*. OvX-F *vs*. OvX+Est-F) and for each treatment group (Veh *vs*. DAZ), mean values for TxB_2 and 6-keto-$PGF_{1\alpha}$ without common superscript are significantly different ($0.0001 \leq P \leq 0.033$). Data are derived from Fig. 25, 26.

4.3. Specific aim 3: To determine the molecular mechanism(s) by which estrogen upregulates constrictor prostanoid function in the rat aorta

4.3.1. The mRNA levels of COX-1, COX-2 and TxS by rat aorta and the effects of OvX and estrogen replacement therapy on their expression

RT-PCR measurements showed that COX-1 mRNA was expressed in both aortic endothelial (Endo) and VSM cells. There were no statistical differences between the male and female COX-1 mRNA expression in either Endo (0.203 ± 0.044 and 0.193 ± 0.043, respectively, $P = 0.879$) or VSM (0.463 ± 0.113 and 0.440 ± 0.087, respectively, $P = 0.879$). OvX and estrogen replacement therapy had no effect on COX-1 mRNA expression in either aortic Endo or VSM (Fig. 27, 28, Table 9).

COX-2 mRNA was expressed in both aortic Endo and VSM. In contrast to the findings for COX-1, the female rat aorta expressed significantly more COX-2 mRNA in both Endo (0.360 ± 0.1, $P = 0.046$) and VSM (0.450 ± 0.064, $P = 0.002$) compared to male (0.050 ± 0.040 and 0.052 ± 0.032, respectively). OvX markedly attenuated the COX-2 mRNA expression by 79% in both Endo (0.077 ± 0.047, $P = 0.031$) and VSM (0.093 ± 0.018, $P = 0.006$), to levels similar to those detected in male. Estrogen replacement therapy restored COX-2 mRNA expression in both Endo (0.410 ± 0.071, P= 0.705) and VSM (0.333 ± 0.062, $P = 0.258$) to levels that did not differ from those in the InT-female aorta (Fig. 28, 29, Table 9).

Table 9. The expression of COX-1, COX-2, TxS, and TP mRNA in vascular endothelium (Endo) or vascular smooth muscle (VSM) cells prepared from male, InT-F, OvX-F and OvX+Est-F rat aortas, measured by RT-PCR.

Endo	COX-1	COX-2	TxS	
InT-F (n=3)	0.20±0.04 [a]	0.36±0.1[c]	0.38±0.12[e]	
Male (n=4)	0.2±0.04 [a]	0.05±0.04[d]	0.1±0.06[f]	
OvX-F (n=4)	0.15±0.04 [a]	0.08±0.05 [d]	0.09±0.07 [f]	
OvX+Est-F (n=3)	0.14±0.04 [a]	0.41±0.07 [c]	0.39±0.02 [e]	
VSM	COX-1	COX-2	TxS	TP
InT-F (n=3)	0.44±0.09[b]	0.45±0.06 [c]	0.25±0.02 [e]	0.44±0.04[g]
Male (n=4)	0.46±0.11 [b]	0.05±.0.03 [d]	0.09±0.02 [f]	0.27±0.03[h]
OvX-F (n=4)	0.49±0.09 [b]	0.09±0.02 [d]	0.07±0.01 [f]	0.26±0.04 [h]
OvX+Est-F (n=3)	0.48±0.05 [b]	0.33±0.06 [c]	0.36±0.12 [e]	0.37±0.03 [g]

Values are expressed as the ratio of specific mRNA to the expression of GAPDH mRNA from the same tissue. Data points represent means ± SE (n, no. of animals). [a-d] For each specific mRNA (COX-1, COX-2, TxS, or TP), mean values for each animal group (male *vs*. InT-F *vs*. OvX-F *vs*. OvX+Est-F) and each treatment (Endo *vs*. VSM) without common superscript are significantly different ($0.05 > P > 0.0001$). Data are derived from Fig. 27, 29, 30.

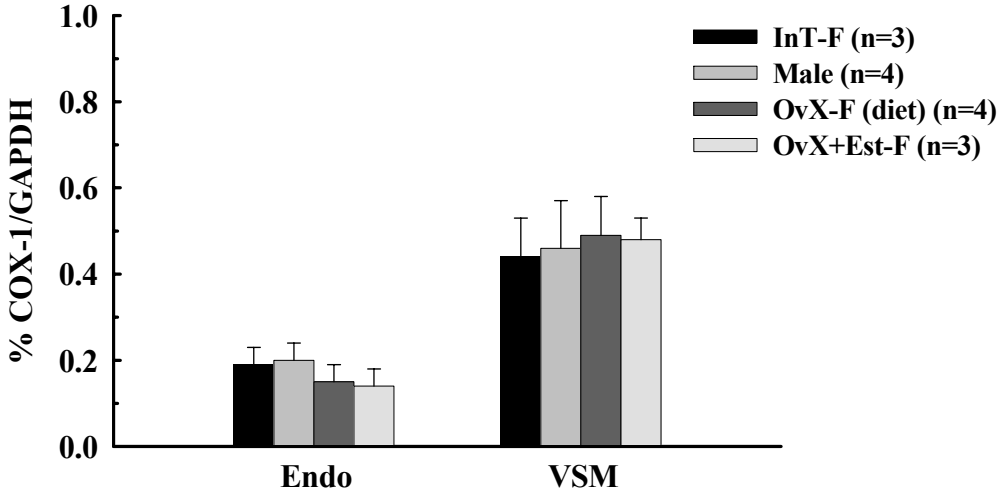

Fig. 27. The expression of COX-1 mRNA levels in vascular endothelium (Endo) and vascular smooth muscle (VSM) cells obtained from male, InT-F, OvX-F and OvX+Est-F rat aortas. The OvX-F rats were fed with phytoestrogen-free diet (diet). Values are expressed as the ratio of COX-1 mRNA to GAPDH mRNA level obtained from the same tissues. Data bars represent means ± SE (n, no. of animals). [a-b], For each group (InT-F *vs*. M *vs*. OvX-F *vs*. OvX+Est-F) and treatment (Endo *vs*. VSM), mean values for COX-1 mRNA expression levels without common superscript are significantly different ($P < 0.05$). No statistically significant differences exist among the four experimental groups in either Endo ($P \geq 0.39$) or VSM ($P \geq 0.69$).

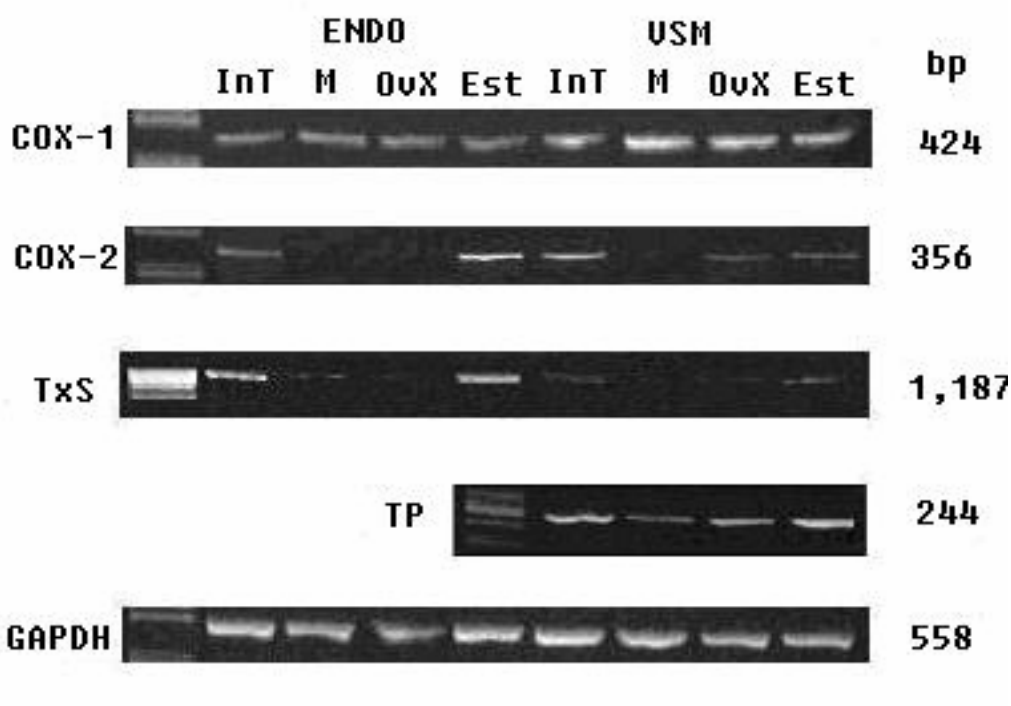

Fig. 28. The RT-PCR gel for COX-1, COX-2, TxS and TP from aortic Endo and VSM cells obtained from male (M), intact female (InT), ovariectomized female (OvX), and estrogen replaced female (Est) rat aortas. The OvX-female rats were fed with phytoestrogen-free diet. The first lane on each gel was the standard DNA ladder. bp, the size of RT-PCR products.

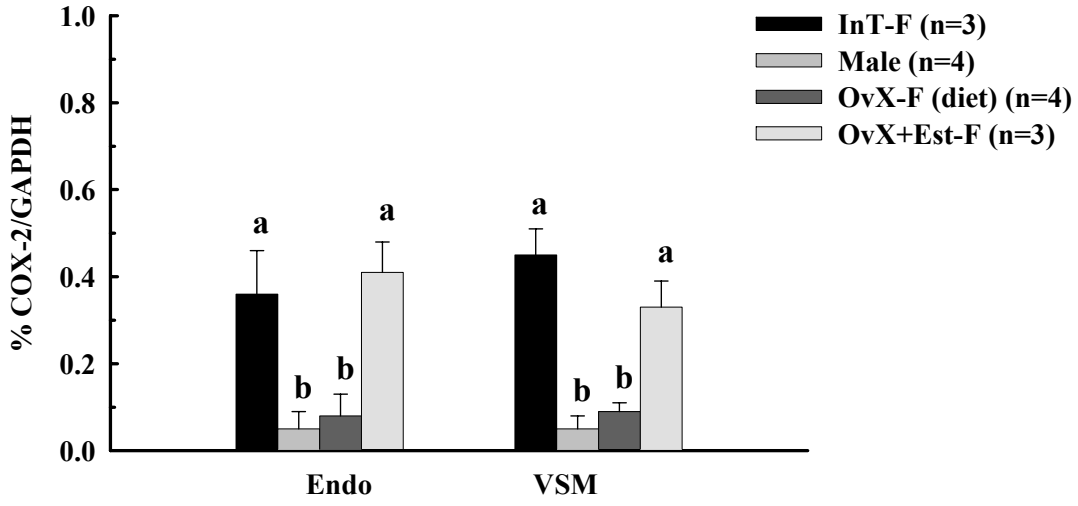

Fig. 29. The expression of COX-2 mRNA levels in vascular endothelium (Endo) and vascular smooth muscle (VSM) cells obtained from male, InT-F, OvX-F and OvX+Est-F rat aortas. The OvX-F rats were fed with phytoestrogen-free diet. Values are expressed as the ratio of COX-2 mRNA to GAPDH mRNA level obtained from the same tissues (%COX-2/GAPDH. Data bars represent means ± SE (n, no. of animals). [a-b,] mean values among the four experimental groups (Male, InT-F, OvX-F and OvX+Est-F) and the two tissue (Endo *vs*. VSM) without common script are significantly different ($0.002 \leq P \leq 0.03$).

Similar to COX-2, TxS mRNA was expressed in both aortic Endo and VSM, and the female rat aorta expressed significantly higher TxS mRNA in both Endo (0.380 ± 0.123, $P = 0.04$) and VSM (0.252 ± 0.015, $P < 0.001$) than the male aorta (0.102 ± 0.063 and 0.090 ± 0.017, respectively). OvX markedly attenuated TxS mRNA expression by 78% in Endo (0.085 ± 0.072, $P = 0.039$) and 72% VSM (0.070 ± 0.010, $P < 0.001$) to levels similar to those observed in male. Estrogen replacement therapy restored TxS

mRNA expression in both Endo (0.387 ± 0.018, P =0.960) and VSM (0.363 ± 0.117, P =0.316) to levels not different from those detected in InT-female (Figure 28, 30, Table 9).

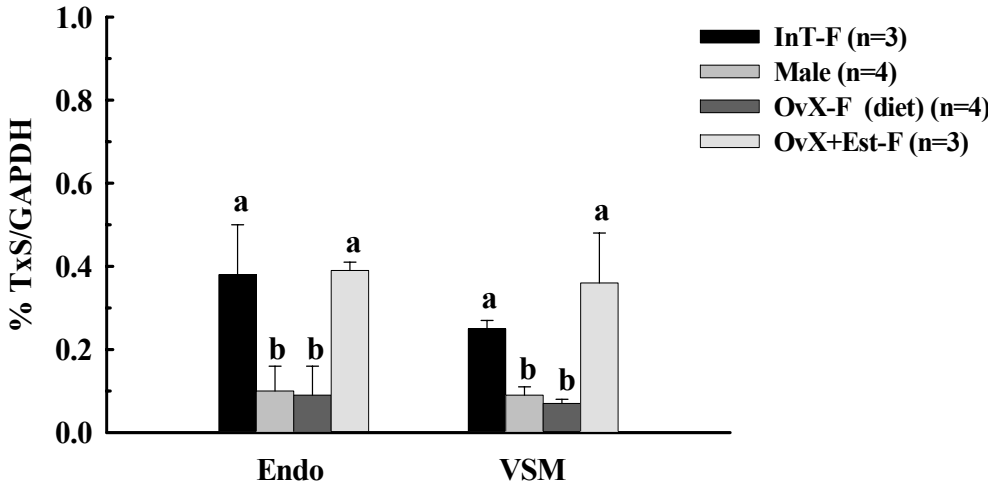

Fig. 30. The expression of TxS mRNA levels in vascular endothelium (Endo) and vascular smooth muscle (VSM) cells obtained from male, InT-F, OvX-F and OvX+Est-F rat aortas. The OvX-F rats were fed with phytoestrogen-free diet (diet). Values are expressed as the ratio of TxS mRNA to GAPDH mRNA level obtained from the same tissues (%TxS/GAPDH). Data bars represent means ± SE (n, no. of animals). [a-b,] mean values among the four groups (InT-F *vs*. M *vs*. OvX-F *vs*. OvX+Est-F) and the two tissues (Endo *vs.* VSM) without common script are significantly different ($0.0003 \leq P \leq 0.04$).

4.3.2. The protein expression of COX-1, COX-2 and TxS enzymes and the effects of OvX and estrogen replacement therapy on enzyme expression

Immunohistochemistry studies provided a way to quantify COX-1, COX-2 and TxS protein expression in the aortic wall. Qualitatively, there was no stain observed on the endothelium from the control group. Very light and similar levels of staining for COX-1 were observed in all four experimental groups. The staining for COX-2 protein

was more obvious on the endothelium of InT-female and OvX+Est-female than of male and OvX-female aortas. Darker staining for TxS was observed on the endothelium of InT-female and OvX+Est-female than on male and OvX-female aortas (Fig. 31). Since protein staining on the thin endothelial layer could not be accurately scored, protein expression was quantitatively scored only in VSM. In VSM, there were no statistically significant differences in COX-1 staining between female (3.6 ± 0.1) and male (3.5 ± 0.2). OvX and estrogen replacement therapy had no effects on COX-1 expression. In contrast, significantly more staining for COX-2 was observed in the female aorta (2.9 ± 0.5) than in the male aorta (1.6 ± 0.4, P = 0.041). OvX markedly attenuated COX-2 protein expression (1.067 ± 0.133, P = 0.011), and estrogen replacement therapy restored the staining for COX-2 (3.067 ± 0.521, P = 0.849) (Fig. 31, Table 10). Similar to the pattern of COX-2 protein expression, significantly more staining for TxS was observed in the female aorta (2.75 ± 0.52) than in the male aorta (1.425 ± 0.253, P = 0.031). OvX markedly attenuated the TxS protein expression (1.20 ± 0.235, P = 0.017), and estrogen replacement therapy restored the staining for TxS (2.05 ± 0.166, P = 0.247) (Fig. 31, Table 10).

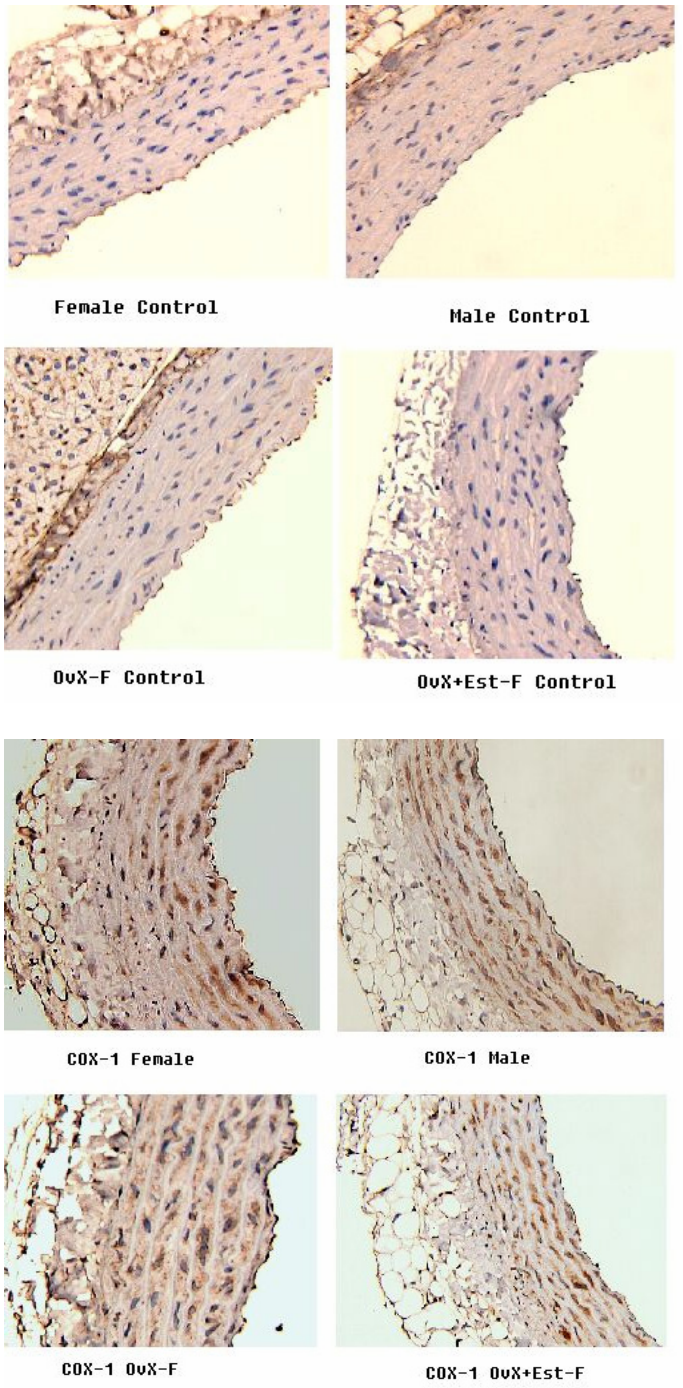

Fig. 31. Immunohistochemical stain (brown color) for COX-1, COX-2 and TxS protein expression in aortic smooth muscle prepared from male, InT-F, OvX-F (fed with phytoestrogen-free diey) and OvX+Est-F rat aortas. Control panel was stained with same procedure but without primary antibody. Blue color showed the counterstain with hematoxylin.

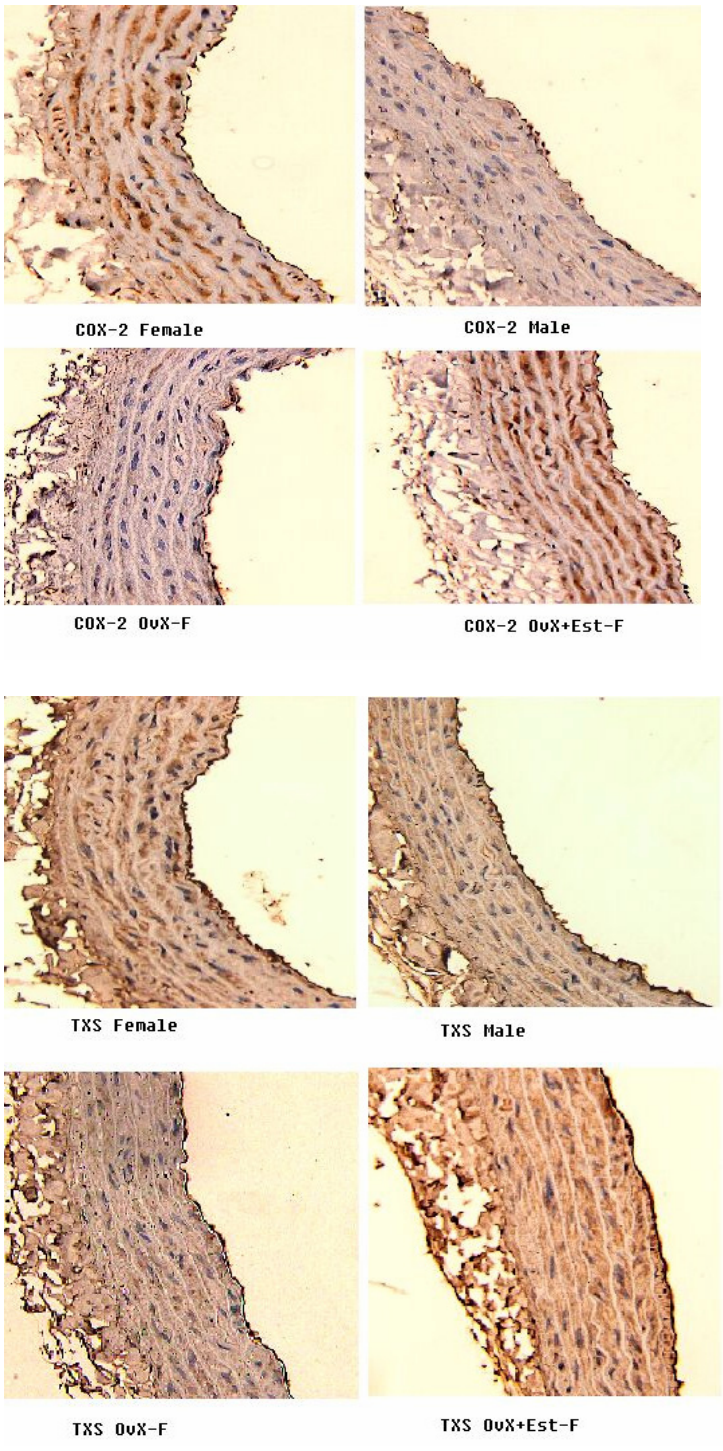

Fig. 31. (Continued)

Table 10. The protein expression of COX-1, COX-2, and TxS in aortic VSM prepared from male, InT-F, OvX-F and OvX+Est-F rat aortas, as measured by immunohistochemistry.

Group	InT-F (n=4)	Male (n=5)	OvX-F (n=4)	OvX+Est-F (n=3)
COX-1	3.6±0.1[a]	3.5±0.2[a]	3.7±0.2[a]	3.2±0.3[a]
COX-2	2.9±0.5[b]	1.6±0.4[c]	1.1±0.1[c]	3.1±0.5[b]
TxS	2.8±0.5[d]	1.4±0.3[e]	1.2±0.2[e]	2.1±0.2[d]

Values are expressed as scores of immunohistochemical stains for COX-1, COX-2, or TxS protein. Data points represent means ± SE (n, no. of animals). [a-e] for each specific protein (COX-1, COX-2 or TxS), mean values without common superscript are significantly different among four experimental groups (male *vs*. InT-F *vs*. OvX-F *vs*. OvX+Est-F) ($0.041 > P > 0.011$).

4.3.3. The expression of the TP mRNA by rat VSM cells and the effects of OvX and estrogen replacement therapy on TP expression

mRNA expression of the TP in aortic VSM was measured by RT-PCR. TP mRNA level was significantly higher in the female (0.51 ± 0.04) than in the male (0.27 ± 0.03, P= 0.004). OvX markedly attenuated TP mRNA expression in the female rat aorta (0.26 ± 0.04, P = 0.010), while estrogen replacement therapy restored receptor expression to a level similar to that of the InT-female aorta (0.37 ± 0.03, P = 0.102) (Fig. 28, 32, Table 10).

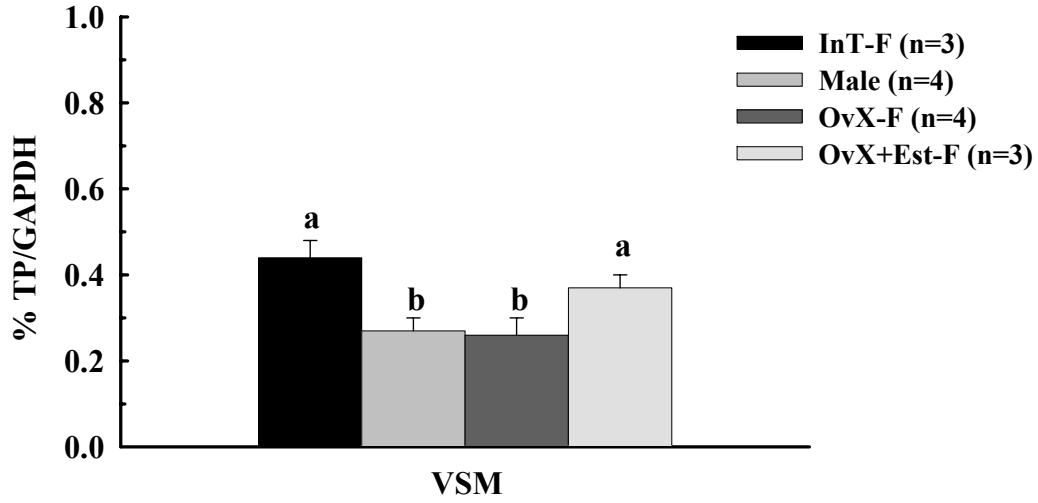

Fig. 32. The expression of PGH_2/TxA_2 receptor (TP) mRNA levels in vascular smooth muscle (VSM) prepared from male, InT-F, OvX-F and OvX+Est-F rat aortas. Values are expressed as the ratio of TR mRNA to GAPDH mRNA level obtained from the same tissue (%TP/GAPDH). Data bars represent means ± SE (n, no. of animals). [a-b,] mean values among the four groups (InT-F *vs*. M *vs*. OvX-F *vs*. OvX+Est-F) without common script are significantly different ($0.008 \leq P \leq 0.019$).

CHAPTER V

DISCUSSION

In the present studies, OvX and estrogen replacement therapy were used to determine the role of estrogen in the regulation of vascular reactivity to VP and constrictor prostanoid potentiation of the vascular responses to VP. Selective COX inhibitors were used to determine the role of COX isozymes in the sex differences in vascular reactivity to VP. To determine the contribution of constrictor prostanoid release to male-female differences in aortic reactivity to VP, basal and VP-stimulated release of TxA_2 and PGI_2 were measured. The effects of estrogen on constrictor prostanoid (TxA_2) release and aortic reactivity to TxA_2 were also studied. Finally, the underlying molecular mechanisms of estrogen action on constrictor prostanoid pathway function were determined by measuring expression of COX-1, COX-2, TxS and TP mRNA by RT-PCR and COX-1, COX-2, and TxS protein production by immunohistochemistry.

The major findings derived from this study are: 1) Estrogen potentiates contractile responses of the female rat aorta to VP by upregulating COX-2, but not COX-1, function; 2) The contractile responses to U-46619 are significantly higher in female than in male rat aorta; and OvX attenuates, whereas estrogen replacement therapy restores, the contractile responses to U-46619 in female rat aorta; 3) VP-stimulated release of both TxA_2 and PGI_2 are higher in female than in male rat aorta; and OvX decreases, whereas estrogen replacement therapy restores, both TxA_2 and PGI_2 release from female rat aorta; 4) Estrogen potentiates COX-2 , TxS and TP message and protein expression in both endothelium and VSM of the female rat aorta.

5.1. Effects of COX inhibitors on contractile responses of female rat aorta to VP

Previous studies established that the contractile responses of the rat aorta to VP are threefold higher in female than in male (70, 224, 225, 226). Similar sex differences in the vascular reactivity to VP have been reported in mesenteric arterioles of anesthetized rats (5) and in the isolated, perfused rat mesenteric vasculature (227, 229). Previous studies also demonstrated that VP-stimulated release of NO is much greater in the male aorta and is responsible for the markedly attenuated responses to VP, compared to the female aorta (224, 225). Interestingly, even in the presence of NOS inhibition, contractile responses to VP are still higher in female, and several studies have revealed that this remaining sex difference in reactivity to VP involves constrictor prostanoids (70, 224). The non-selective COX inhibitor Indo and the TP antagonist SQ 29,548 both attenuated contractile responses of the female but not the male rat aorta to VP to the same extent (70), which established that constrictor prostanoids play a significant role in potentiating contractile responses of the female but not the male systemic vasculature to VP. However, these studies did not examine the underlying mechanisms of these sex differences in detail at the cellular or molecular levels.

To further determine the role of constrictor prostanoids in VP-induced contraction of the female rat aorta, the effects of COX inhibitors were examined in the present study. The non-selective COX inhibitor Indo significantly attenuated the contractile responses of the female rat aorta to VP by 43%. Interestingly, the chemically dissimilar COX-2-selective inhibitors NS and NA both attenuated contractile responses to VP to a similar extent as Indo (41% and 46% respectively), which strongly suggests

that COX-2 but not COX-1 mediates at least in part, the constrictor prostanoid potentiation of the contractile responses of the female rat aorta to VP. Thus, the present study clearly establishes the involvement of COX-2 in the constrictor prostanoid-mediated potentiation of the contractile responses of the systemic vasculature of the female but not the male to VP.

COX-1 and COX-2 are two isoforms of the cyclooxygenase enzyme. Different genes encode these isoforms (131, 132). COX-1 is the constitutive isoform present in many cells and is a housekeeping enzyme (219, 255). Conversely, COX-2 is an inducible isoform activated by inflammatory agents (116, 186); however, COX-2 is also constitutively expressed in some tissues such as the kidney, lung, spinal cord and heart (180, 186, 254), and may play a role in regulating normal renal function and blood flow. The present study is the first to provide clear evidence that COX-2 is expressed constitutively in the rat aorta under normal physiological conditions, and is involved in the regulation of systemic vascular function in the female but not the male rat.

5.2. Effects of OvX and estrogen replacement therapy on contractile responses of female rat aorta to VP

To determine the role of estrogen in the regulation of constrictor prostanoid pathway function in the female rat aorta, the effects of OvX and estrogen replacement therapy on vascular reactivity to VP and prostanoid function were determined. Plasma 17β-estradiol concentrations measured in the present study are consistent with those of previous studies (220, 250). Plasma concentrations of 17β-estradiol were attenuated

drastically in OvX-female rats, and did not differ from those of male rats. Estrogen replacement therapy restored plasma estrogen concentrations of OvX-female rats to levels not significantly different from those of InT-female rats (Table 4).

OvX markedly attenuated contractile responses to VP in the female rat aorta on phytoestrogen-free diet, and abolished the inhibitory effects of both Indo and NS. In contrast, long-term (3 – 4 weeks) estrogen replacement therapy with 17β-estradiol in OvX-female rats completely restored both the contractile responses to VP and the inhibitory effects of Indo and NS. These results clearly establish that constrictor prostanoid potentiation of the contractile responses of the female rat aorta to VP is strongly dependent upon the action of estrogen on the vascular wall.

Estrogen receptors have been identified in both the endothelium (7, 38, 41) and VSM (7, 173, 181); thus, the potentiating effect of estrogen on constrictor prostanoid function in the female aorta may involve actions on the endothelium and/or VSM. In the present study, pharmacological blockage of the prostanoid pathway at the level of COX suggests that the potentiating effect of estrogen involves upregulation of COX-2 and the subsequent constrictor prostanoid pathway, thereby regulating both the release and action of the constrictor prostanoids.

5.3. Effects of estrogen on prostanoid biosynthesis pathway

The present study revealed that VP stimulated production of TxA_2 and PGI_2 from both male and female rat aorta in a concentration-dependent manner, and that VP-stimulated release of TxA_2 and PGI_2 was significantly higher in female than in male.

OvX attenuated and estrogen replacement therapy restored VP-stimulated release of TxA_2 and PGI_2 from the female rat aorta. These findings demonstrate that estrogen potentiates VP-stimulated release of TxA_2 and PGI_2 in female aorta. Numerous previous studies support the present finding that estrogen enhances vascular prostanoid production. For example, pretreatment of isolated, blood-perfused lungs of juvenile female sheep with estradiol enhances the production of TxA_2 (235). Similarly, in the isolated, exogenous arachidonic acid perfused rat lung, production of PGI_2 and TxA_2, which reflects COX activity, varies during the course of the estrous cycle and peaks during the estrogen surge at proestrous (10). Further, estrogen treatment enhances TxA_2 production in rat endothelial cells (262) and PGI_2 production in both rat VSM cells (34) and ovine fetal pulmonary artery endothelial cells (217). However, the present study is the first to establish the importance of estrogen in the regulation of vascular TxA_2 production by the systemic vasculature.

The present study also revealed that the non-selective COX inhibitor, Indo, and the COX-2-selective inhibitor, NS, both attenuated the release of TxA_2 to a similar extent (91% and 90%, respectively) by the female aorta. This finding is consistent with the equipotent attenuating effects of both Indo and NS on the contractile response to VP in the female aorta; thus, the present study is the first to demonstrate that estrogen potentiates VP-stimulated release of prostanoids by the female rat aorta, at least in part, by upregulating COX-2 function.

In contrast to the findings on VP-stimulated release of TxA_2 and PGI_2, basal release of both TxA_2 and PGI_2 did not differ among the four experimental groups. One

explanation for this difference is that the enzymes involved in the biosynthesis of TxA_2 may exist in different cellular compartments and may function differently under basal and agonist-stimulated conditions, or that the enzymes have activated and inactivated forms. For example, COX-1 and COX-2 both exist in the endoplasmic reticulum and in the nuclear envelope (221); however, recent studies show that COX-2 also exists in the plasma membrane, complexed with caveolin-1 (149), and the concentration of COX-2 is twofold greater in nuclear envelope than in endoplasmic reticulum (166), especially localized at the perichromatin zone (187). Thus, it is possible that the potentiating effect of estrogen on the prostanoid biosynthesis pathway is different under basal and agonist-stimulated conditions. Estrogen and agonists may work synergistically, or act differently than under basal conditions, to stimulate the expression of COX-2 that locates in a particular cellular compartment. It is also possible that estrogen-induced synthesis of enzyme molecules needs to be activated by an agonist, such as VP, to exert its catalytic function in TxA_2 biosynthesis pathway. Further experiments should be done to elucidate the interaction of estrogen and agonists involved in prostanoid biosynthesis and the subcellular location of COX-2 stimulation.

It is well known that estrogen can stimulate expression of eNOS and enhance NO release (98, 126, 259). The balance between vasodilation and vasoconstriction is very important for the regulation of vascular tone. The higher release of NO in the female vasculature (121, 129) may result in the need for mechanism(s) to counterbalance the vasodilator effect of NO; the co-existence of NOS and COX-2 in caveolin imply that

estrogen may play a role in counterbalancing the NO-vasodilatory mechanism by stimulating the production of constrictor prostanoids.

TxA_2 causes platelet aggregation, and is a vasoconstrictor. In contrast, PGI_2 prevents platelet aggregation and is a vasodilator. The balance between TxA_2 and PGI_2 is very important in the regulation of homeostasis of the circulatory system (29, 245); and an imbalance between TxA_2 and PGI_2 is associated with the pathogenesis of some cardiovascular diseases such as pulmonary hypertension and atherosclerosis (37, 76, 106, 244). It is reported that injury-induced vascular proliferation and platelet activation are enhanced in mice that are genetically deficient in the PGI_2 receptor, but are depressed in mice genetically deficient in the TP or treated with a TP antagonist (36).

TxA_2 and PGI_2 are both derived from the same prostanoid metabolic intermediate PGH_2, a COX product, by the actions of TxS and prostacyclin synthase, respectively, on PGH_2. The present study uniquely revealed the dynamic characteristics of the prostanoid pathway as a whole, and that an interrelationship exists between TxA_2 and PGI_2 synthesis within the prostanoid metabolic pathway. In the presence of the TxS inhibitor DAZ, VP-stimulated release of TxA_2 was markedly attenuated, whereas, the production of PGI_2 was increased. This finding reveals that with inhibition of the TxS pathway, accumulation of the common upstream intermediate PGH_2 then alters the activity of the prostacyclin synthase pathway, enhancing the production of PGI_2. Since PGI_2 is an important local vasodilator and inhibitor of platelet aggregation, the present findings imply that TxS inhibitors may be an effective therapy for constrictor prostanoid-

mediated systemic vascular diseases, not only by inhibiting the production of vasoconstrictor TxA_2, but also by potentiating the production of vasodilator PGI_2.

Another interesting finding about the effects of DAZ on the release of TxB_2 and 6-keto-$PGF_{1\alpha}$ is that DAZ tremendously increased 6-keto-$PGF_{1\alpha}$ release in male (298%) and OvX-female (178%) aortas, but only increased 6-keto-$PGF_{1\alpha}$ release by 66% in InT-female and by 42% in OvX+Est-female aortas. It was reported that prolonged use of combined oral contraceptives was associated with decreased production of $PG1_2$ (268), and estrogen decreases PGI_2 production in both intact and castrated male rats (39). Thus, the increased release of 6-keto-$PGF_{1\alpha}$ in the presence of DAZ, especially the huge increase in male and OvX-female aortas implies that there may exist the combined inhibitory effect of TxA_2 and estrogen on PGI_2 production, perhaps via regulating PGI_2 synthase activity. So that in the presence of DAZ and loss of estrogen, the PGI_2 synthesis is dramatically increased.

5.4. Effects of estrogen on vascular reactivity to thromboxane

The present study demonstrates that estrogen not only enhances the activity of COX-2 and the release of constrictor prostanoids, but also potentiates vascular reactivity to the constrictor prostanoid TxA_2. Contractile responses to the TxA_2 analog U-46619 were significantly higher in female than in male aorta. This finding is consistent with the findings in the isolated rat pulmonary vasculature (65). In both past and present studies, OvX and phytoestrogen-free diet abolished and estrogen replacement therapy restored the contractile responses of the female vasculature to U-46619. These results reveal that

estrogen enhances contractile responses of female aorta to the constrictor prostanoid TxA_2. The underlying mechanisms may include: 1) estrogen-induced increases in the density of TP in rat aortic VSM cells; 2) U-46619-stimulated release of vasoactive substance(s) from the vascular endothelium, which may be upregulated by estrogen; or 3) estrogen-enhanced downstream signal transduction mechanisms is involved in TxA_2-stimulated VSM contraction. A previous study using cultured rat aortic VSM cells reported that the total number of TxA_2 binding sites per cell is higher in female than in male VSM cells (155); however, the investigators did not examine reactivity of the female aorta to TxA_2. Therefore, it is possible that the greater contractile responses to U-46619 in the female rat in the present study, especially in Endo- aortic rings, may involve upregulation of TP expression by estradiol in VSM cells. Concentration-response curves with paired Endo+ and Endo- aortic rings in the present study revealed that deletion of the endothelium increased contractile responses to U-46619 in both male and female, but the effect of de-endothelialization did not differ between male and female. The increase in contractile responses to U-46619 after removal of the endothelium may be due to the loss of endothelial vasodilators, especially NO; however, this mechanism does not appear to contribute to sex differences in the reactivity of the rat aorta to U-46619. Studies with human omental arteries also showed that neither inhibition of NOS with N-nitro-L -arginine nor removal of the endothelium affected contractile response to U-46619 (247). Finally, estrogen also may influence downstream signal transduction mechanisms involved in the thromboxane-induced VSM contraction.

The present study demonstrated that the potentiating effects of estrogen on contractile responses of the female aorta to VP involve upregulation of COX-2 activity, thereby enhancing production of TxA_2 and potentiating vascular reactivity to TxA_2. The greater contractile responses of the female aorta to VP also may be due to the upregulation of VP receptors in VSM cells by estrogen. Estrogen significantly increases the expression of VP $V_{1\alpha}$ receptor mRNA in the preoptic area of young OvX rats (71). Similarly, estrogen increases VP V_1 receptor expression in rabbit myometrium and in rat mesenteric arterioles (152, 229). In the latter study, upregulation of VP V_1 receptors was correlated with an increase in the vasoconstrictor responses to VP in the isolated, perfused mesenteric vascular bed. Estradiol also increases the maximal response to VP and the density of VP binding sites in the mesenteric vasculature of male rats (229). In present study, OvX reduced maximal contractile response to VP by 55%, which was greater than the effects of Indo or NS (43% and 41% respectively). Thus, it is possible that OvX not only decreases constrictor prostanoid function, but also reduces the expression of VP binding sites by aortic VSM cells.

5.5. Effects of phytoestrogens on vascular responses to VP and TxA_2

The present study revealed that depletion of phytoestrogens from rat chow attenuates the contractile response to VP and TxA_2 in OvX-female aorta. This finding is consistent with previous studies, which demonstrated that phytoestrogens could interact with the estrogen receptor, and exert estrogen-like effects (2, 40, 59). Indeed, phytoestrogens were reported to interact with COX and thereby regulate prostanoid

biosynthesis (54). Thus, in present study, phytoestrogens are likely responsible for the continued release of TxA_2 in the OvX-female rat aorta, and masking the effects of OvX on vascular reactivity to VP and U-46619; however, adequate testing of this hypothesis will require further studies.

5.6. Effects of estrogen on expression of COX and TxS mRNA and protein

In the present study, the expression of COX-2 mRNA and protein were higher in female than in male rat aorta. OvX attenuated and estrogen replacement therapy restored the expression of both mRNA and protein. In contrast, estrogen had no effect on expression of COX-1 mRNA or protein in the female rat aorta. The present findings are supported by previous studies, which suggest that estrogen may regulate vascular reactivity by upregulation of COX-2 and prostanoid production. For example, in cultured vascular endothelial cells, estrogen induces both COX-2 gene expression and new COX-2 protein synthesis, resulting in increased production of PGI_2 and PGE_2 (189). Similarly, estrogen enhances PGI_2 production in human umbilical vein endothelium cells by increasing COX-2 but not COX-1 expression (4). Thus, both past and present studies involving measurements vascular reactivity, prostanoid release, and gene expression consistently support the idea that estrogen enhances prostanoid-potentiated contractile responses to VP in female rat aorta by upregulating COX-2 activity.

The present study also demonstrated that estrogen enhances the expression of TxS mRNA and protein expression in the female aorta. Although TxS inhibition in some previous studies failed to attenuate constrictor prostanoid-potentiated vasoconstriction

(70), as discussed above, the use of a TxS inhibitor will result in the accumulation of the upstream intermediate, PGH_2, which is also a vasoconstrictor and acts on the same receptor as TxA_2. Thus, the failure of the TxS inhibitor to attenuate vasoconstriction establishes the involvement of PGH_2, but cannot exclude the involvement of TxA_2 in potentiating contractile responses of the female aorta to VP.

The present study also revealed that TxS mRNA is expressed in both endothelium and VSM, and that OvX attenuated and estrogen replacement therapy restored TxS mRNA expression in both endothelium and VSM. Estrogen also enhanced TxS protein expression both in the endothelium and VSM. Although previous studies have already revealed that COX-2 mRNA and protein are expressed in cultured vascular endothelium (182, 187, 189) and cultured VSM cells (182), the present study is the first to establish the expression of TxS mRNA and protein in both the endothelium and VSM of intact systemic blood vessels. Previous studies reported the production of TxA_2 from cultured vascular endothelium but not VSM (79, 81); however, preliminary studies in this doctoral research revealed that TxA_2 is released from both Endo+ and Endo- (VSM) aortic rings (data not shown), which is consistent with the molecular findings that both COX-2 and TxS are expressed in both endothelium and VSM cells of the female rat aorta.

5.7. Effects of estrogen on thromboxane receptor expression

In the present study, it was determined that TP mRNA is expressed in aortic VSM cells, and that expression is higher in female than in male aorta. Further, OvX

attenuates and estrogen replacement therapy restores TP mRNA expression in VSM cells. These findings are consistent with the present finding that contractile responses to U-46619 are higher in female than in male aorta, and are attenuated by OvX in the female aorta and with the previous finding that the contractile responses to U-46619 in isolated rat lung are enhanced by estrogen (65). Thus, the present findings reveal that estrogen potentiates contractile responses of the female aorta to VP by enhancing the production of and the reactivity to TxA_2. Further, this effect of estrogen involves upregulation of both message and protein expression of COX-2 and TxS.

5.8. Clinical significance of present study

Under normal physiological conditions, there are a variety of male-female differences in cardiovascular function. For example, basal blood pressure (125, 261), production of NO (91, 121), and vascular reactivity to VP, PE, U-46619 (65, 70, 117), all exhibit sexual dimorphism. The results from the present study advance our knowledge of male-female differences in cardiovascular function under normal condition and the role of estrogen in the sexual dimorphism present in cardiovascular function. Estrogen modulates prostanoid pathway function in the systemic vasculature by upregulating the expression of both COX-2 and TxS, resulting in increased production of TxA_2 and PGI_2. It also upregulates the expression of TP and thereby potentiates vascular reactivity to the constrictor prostanoids TxA_2 and PGH_2.

The findings of the present study are consistent with the extensive epidemiological data that reveal the existence of prominent sexual dimorphism in the

incidences of primary vascular diseases that involve excessive vasoconstriction. Primary pulmonary hypertension (73, 252), migraine headache (194), and Raynaud's disease (14, 25, 87, 251) all occur in premenopausal women at rates as much as fourfold higher than in men. Further, an association has been reported between Raynaud's disease and these other afflictions, suggesting that a common mechanism of vasospasm may be involved (236). Elevated vascular production of TxA_2 may be the common mediator of vasospasm in all of these diseases. The production of this constrictor prostanoid is increased in patients with Raynaud's disease (208), migraine headache (127), and pulmonary hypertension (37). Animal studies also have revealed the relationship between TxA_2 and the development of these diseases (30, 67). Further, TxS inhibitors such as CGS13080 and dazoxiben have been used successfully in the treatment of primary pulmonary hypertension and Raynaud's disease (18, 198).

The higher incidences of peripheral vascular diseases in premenopausal women than in men suggest that estrogen and/or other ovarian steroids may be involved in the pathogenesis of primary vascular diseases in women. Indeed, the recent Heart and Estrogen-progestin Replacement Study (HERS) reported no overall benefit of estrogen replacement therapy, and untoward cardiovascular events, including venous thrombosis, actually increased significantly in the first year of the study (94, 95, 105). Furthermore, oral contraceptive use in young women is also associated with an increased risk of thrombosis (17, 52) and acute myocardial infarction (AMI) (111). The increased production of TxA_2 in the systemic vasculature with estrogen replacement therapy, as established in the present study, may underlie these deleterious effects of estrogen on the

cardiovascular system. Indeed, postmenopausal women undergoing estrogen replacement therapy have elevated urinary levels of TxA_2 (249). Interestingly, the present study demonstrated that estrogen upregulates the release of TxA_2 by increasing the expression of message for COX-2 and TxS, and the expression of COX-2 and TxS protein. These findings raise the intriguing possibility that the deleterious effects of estrogen on vasculature function and/or thrombosis, suggested by earlier (94, 95, 105) and more recent HERS II epidemiological studies (113, 215), may involve the effect of estrogen to potentiate activity of TxA_2 and the constrictor prostanoid pathway function.

VP is a potent vasoconstrictor that increases blood pressure and systemic vascular resistance. It has been used in the treatment of vasodilatory shock seen in sepsis, systemic inflammatory response, hypovolemia, cardiac arrest, polytrauma (35, 134), and for esophageal variceal bleeding in patients with liver cirrhosis (142). As an antidiuretic hormone, it is also been used to control the loss of water associated with diabetes insipidus (160). However, clinical trials assessing the safety and efficacy of VP use are limited, and male-female differences in the responses to clinical uses of VP are unknown. The present study provides clear evidence that vascular responsesiveness to VP is remarkably higher in females than in males, and that estrogen potentiates constrictor responses to VP in the female vasculature by upregulating COX-2 function and VP-stimulated TxA_2 production. Since TxA_2 produces vasoconstriction and platelet aggregation, TxA_2-mediated effects of VP administration need to be considered, especially in female patients. Indeed, it was reported that a 46 year-old female patient who recovered from acute respiratory distress syndrome following development of

septic shock, experienced severe ischemic necrosis of the skin and soft tissue surrounding the catheter site following administration of VP to treat the septic shock (115). The present study draws attention to possible side effects of VP as a potent vasoconstrictor used in clinical settings. The prominent male-female differences in VP-induced vasoconstriction and activation of the TxA_2 pathway suggest that different strategies may be needed for clinical uses of VP in male versus female patients. VP is also an important mediator during the development of heart failure. Plasma VP concentrations are significantly and chronically elevated in patients with heart failure, particularly in those with significant cardiac decompensation and hyponatremia (82, 83). VP contributes to the development of heart failure by preventing free water excretion and by inducing vasoconstriction. VP antagonists have been used in the treatment of heart failure (82). The findings of the present study suggest an additional role of VP in the development of heart failure which involves VP-stimulated constrictor prostanoids, particularlly in both male and female patients, and also imply that endogenous elevations in VP levels may have more deleterious effects in females than in males because of the combination of direct and TxA_2-mediated effects on the cardiovascular system.

CHAPTER VI

CONCLUSIONS

In conclusion, the present studies demonstrated that sex differences exist in the contractile responses of the Sprague-Dawley rat aorta to VP and U-46619, and that estrogen potentiates contractile responses of the female rat aorta to VP and U-46619 by upregulating the expression of COX-2, TxS and TP mRNA, as well as the protein expression of COX-2 and TxS. These effects of estrogen enhance both the production of and reactivity to TxA_2 by the female rat aorta.

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